PURINERGIC SIGNALING AND INFLAMMATION

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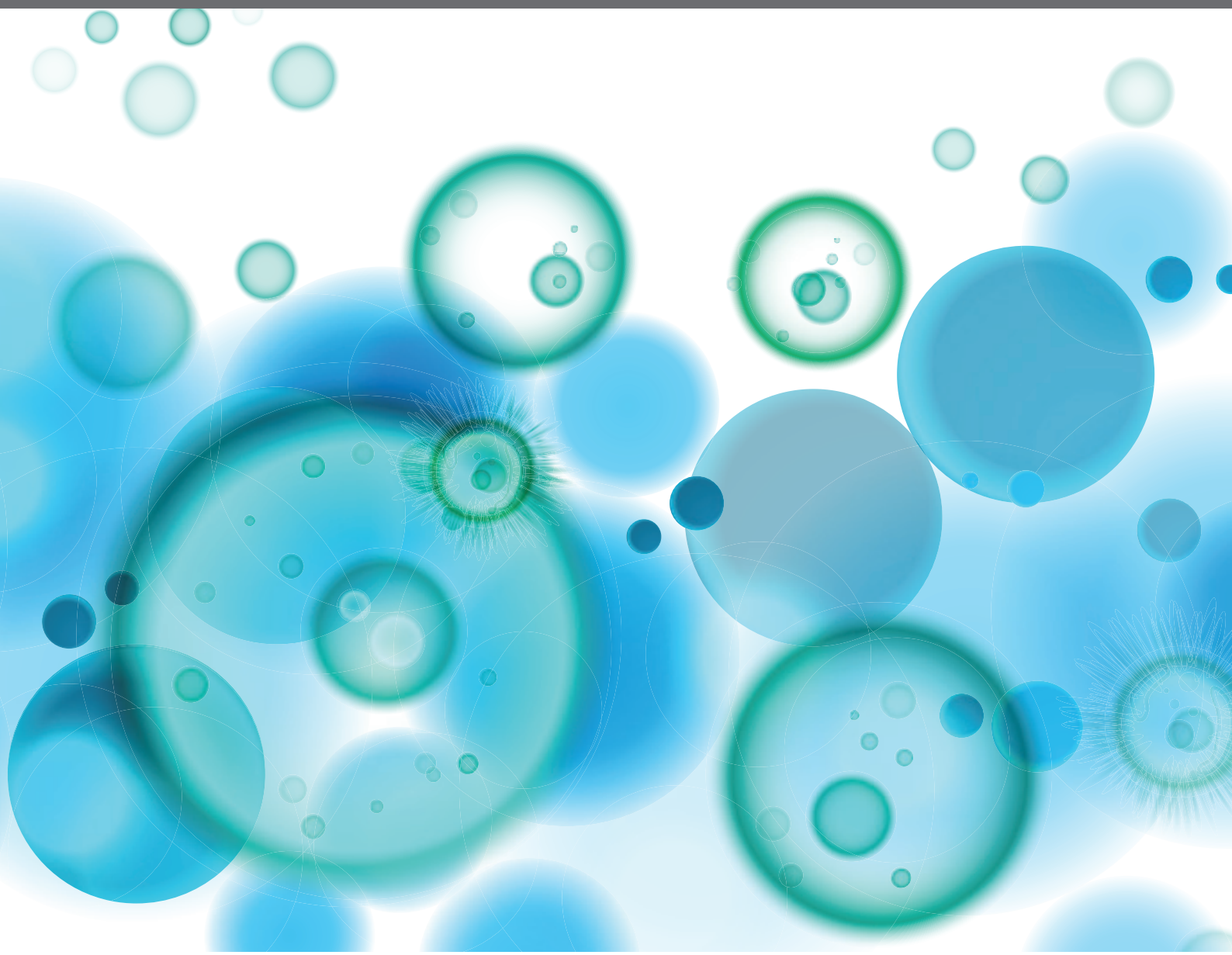

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PURINERGIC SIGNALING AND INFLAMMATION

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EDITORIAL
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Editorial: Purinergic Signaling and Inflammation

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Editorial on the Research Topic

Purinergic Signaling and Inflammation

Purine nucleotides and nucleosides are essential building blocks for cellular energy. Extracellular nucleotides and nucleosides signaling is increasingly recognized to control many other human physiological processes, including the pathogenesis of inflammatory diseases (**Figure 1**, material from Idzko et al.) (1). Adequate inflammatory responses are critical to fighting against invading pathogens and recovering from tissue injury. However, unresolved or chronic inflammation can cause tissue injury and disease pathogenesis (2, 3). The proper control of tissue inflammation requires synergistic action of many different pathways, with purinergic signaling playing diverse and important roles in this process (4–6). In this special Research Topic, the interaction between purinergic signaling and inflammation was highlighted by several original, review, opinion, and perspective articles.

Purinergic signaling orchestrates mucosal inflammation. An opinion article from Antonioli et al. encapsulates the contribution of adenosine system in many aspects of inflammatory bowel diseases (IBD), including intestinal inflammation, abdominal pain, and enteric dysmotility. Preclinical studies indicate that targeting A2A and A3 adenosine receptor has great therapeutic potential for IBD. While activation of those two adenosine receptors by selective agonists is beneficial in attenuating many aspects of IBD, this article highlights the need to develop novel, selective ligands on adenosine receptors. Relatedly, a mini-review article by Vuerich et al. describes how purinergic signaling controls gut inflammation. Indeed, many studies have indicated that therapeutic targeting of ATP (P1) receptor, adenosine (P2) receptor, and ENTPD1/CD39, and/or ecto-5′-nucleotidase (CD73) can directly modulate intestinal inflammation. The mini-review provides a concise overview of the current knowledge of purinergic-based therapy for IBD. Besides, an original article by Libera et al. elaborates on the role of CD39 and CD73 in IBD. The study compares the expression of CD39 and CD73 in T cell populations that include CD4, CD8, and γδ T cells in peripheral blood, as well as mucosal tissue from healthy individuals and IBD patients. Peripheral T cells have a $CD39^{low}CD73^{high}$ phenotype with high levels of IL-17A and IFNγ, while gut mucosal T cells have a $CD39^{high}CD73^{low}$ phenotype and low expression of IL-17A, IFNγ, and IL-10. These results suggest that CD39 and CD73 might be important for the phenotypic adaptation of T cells in the gut mucosal environment. Extending this to another organ system, the review article by Li et al. highlights the important role of adenosine signaling in the crosstalk between hypoxia and inflammation in lung injury. Hypoxia and inflammation are tightly linked together

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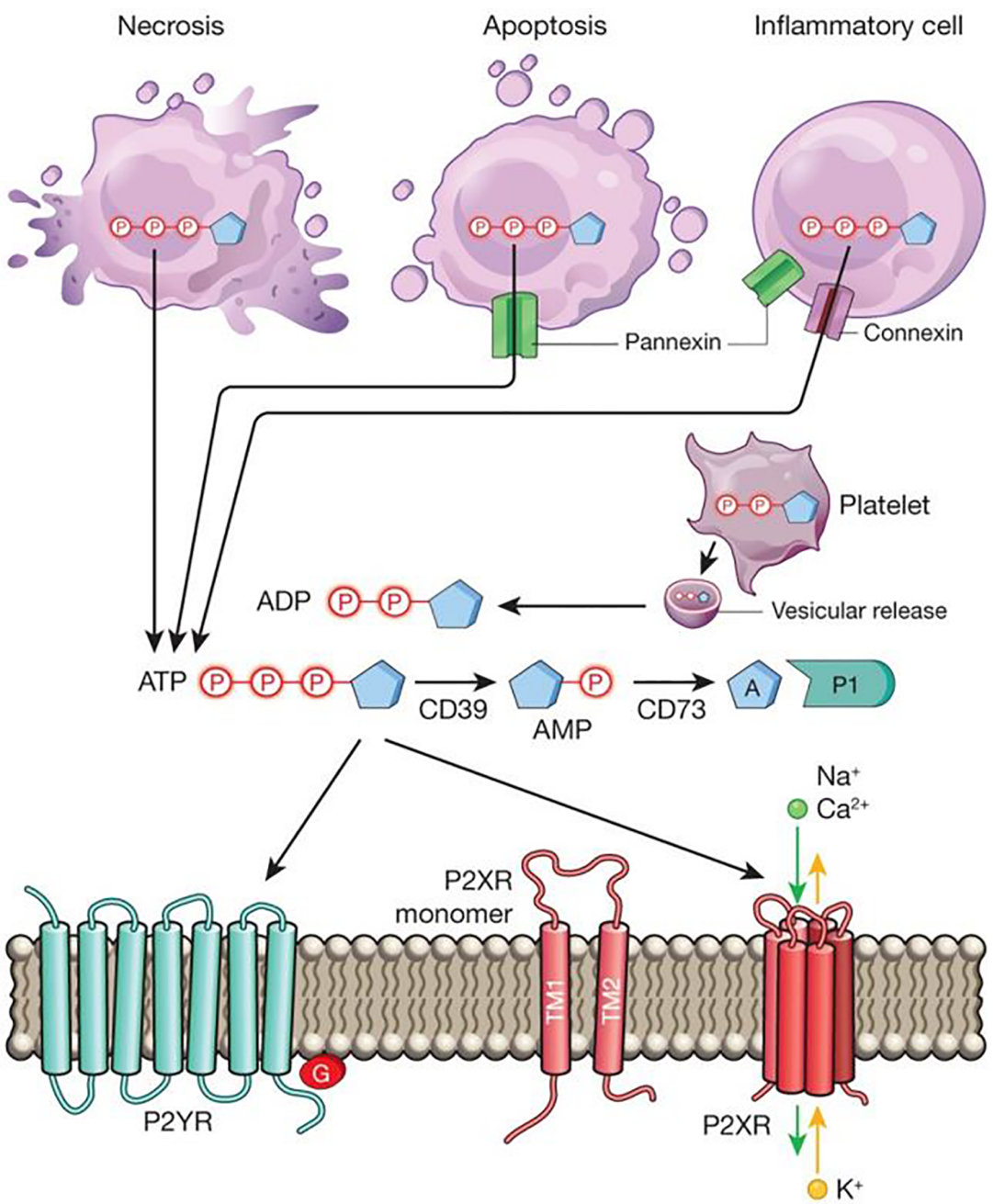

FIGURE 1 | Extracellular nucleotide release and signalling during inflammation. During inflammation, multiple cell types release nucleotides, for example ATP or ADP, from their intracellular compartments into the extracellular space. Nucleotides can be released during mechanical injury, necrosis, apoptosis or inflammatory cell activation. Several molecular pathways have been implicated in this process, such as vesicular ADP release from platelets, pannexin-mediated ATP release during apoptosis, and connexin- or pannexin-mediated ATP release from inflammatory cells, such as neutrophils. Extracellular nucleotides function as signalling molecules through the activation of purinergic P2 receptors. These receptors can be grouped into metabotropic P2Y receptors (P2YRs; GPCRs with seven transmembrane-spanning motifs) or ionotropic P2X receptors (P2XRs), which are nucleotide-gated ion channels. Each P2XR is formed by three subunits (P2XR monomers), each of which consists of two transmembrane regions, TM1 and TM2. Binding of three molecules of ATP to the assembled P2X channel causes opening of a central pore. These conformational changes allow for flux of ions such as sodium (Na+), calcium (Ca2+) and potassium (K+) across the membrane. ATP signalling is terminated by the enzymatic conversion of ATP to adenosine through the ectonucleoside triphosphate diphosphohydrolase CD39 (conversion of ATP/ADP to AMP) and the ecto-5′-nucleotidase CD73 (conversion of AMP to adenosine). Similar to ATP, adenosine (A) functions as an extracellular signalling molecule through the activation of purinergic P1 adenosine receptors. Material from: Idzko et al. (1). Reprinted with permission.

(7). Hypoxia signaling results in the activation of adenosine signaling *via* induction of A2A adenosine receptor and A2B adenosine receptors (8). Among these, the HIF/adenosine axis provides lung protection in acute respiratory distress syndrome though it also promotes inflammation and injury in chronic lung disease (9). Moreover, this article discusses the strategies to therapeutically target the adenosine signaling pathway in lung disease.

Inflammation is commonly observed in cancer and purinergic signaling is crucial in many aspects of cancer development. The review article by Steingold and Hatfield highlights the evidence to support targeting hypoxia-A2A adenosine receptor pathway to release the immunosuppression of anti-tumor T cells. The activation of hypoxia-adenosine-A2A axis in cancer leads to immunosuppression by inhibiting the effector function of T cells. Preclinical and clinical studies suggest that inhibitors targeting A2A, CD39/CD73, or hypoxia signaling can control cancer development when combined with immune checkpoint inhibitors. Moreover, a mini-review by Hamarsheh and Zeiser discusses the contradictory roles of NLRP3 inflammasome in cancer. NLRP3 inflammasome is canonically activated by danger-associated molecular patterns when concurrently exposed to a secondary signal, such as hypoxia, reactive oxygen species, or P2X7R activation. The article concludes that NLRP3 inflammasome acts as a double-edged sword in cancer and future studies should dissect the functional determining factors.

Purinergic signaling is important in modulating immune cell functions during inflammation. The review article by Ferrari et al. highlights how purinergic signaling shapes eosinophil phenotype to elicit pro-inflammatory or anti-inflammatory responses during homeostasis or pathological conditions. The article summarizes the diverse functions of eosinophils in the fight against invading microorganisms and allergic responses. Specifically, the article elegantly highlights how P2 receptors and P1 receptors differentially regulate eosinophil migration and function to exert pro-inflammatory or anti-inflammatory responses during tissue inflammation. The article concludes that P2 receptor inhibitors are potential therapeutic candidates in eosinophilic diseases, while further understanding about nucleotide stimulation of eosinophils in other inflammatory conditions such as cancer is needed. P2 receptor signaling is also crucial to the regulation of T cell function. A mini-review article by Ledderose and Junger describes the convergence of metabolic and purinergic signaling in the modulation of T cell function in host immune defense and inflammatory disorders. ATP accumulation is commonly observed during tissue injury and ATP can bind to P2X and P2Y receptors to modulate T cell function. This review provides a concise overview of how different P2 receptors interact with mitochondria to govern multiple aspects of T cell functions including T cell quiescence, migration, and formation of the immune synapse. Finally, the perspective article from Grassi further highlights the functions of P2X7 receptor in T cell regulation. The article concludes that P2X7 guides the development of γδ T cell in the thymus and promotes the development of Th1 and Th17 responses, conversion of Treg to Th17 cells, and cell death of Tfh cells in peripheral lymphoid organs. Demonstrating a particular role in intestinal immune homeostasis, microbiota-derived extracellular ATP results in P2X7 activation to induce cell death of effector T cells and, in turn, attenuates murine model of colitis.

The articles included in this Research Topic provide an inspiring overview of the interplay between purinergic signaling and inflammation. The collection of articles in the Research Topic also shed light on the importance and complexity of purinergic signaling in different disease settings, such as mucosal inflammation and cancer. Taken together, the papers presented in this Research Topic indicate that new therapeutic developments targeting purinergic signaling are needed to harness this pathway for the treatment of tissue inflammation.

AUTHOR CONTRIBUTIONS

XY drafted the manuscript. DF, MI, and HKE edited the topic and revised the manuscript. TM, YW, AC, M-FD, and SEE edited the manuscript. All authors contributed to the article and approved the submitted version.

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PERSPECTIVE
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The P2X7 Receptor as Regulator of T Cell Development and Function

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Unique structural features characterize the P2X7 receptor with respect to other P2X family members. Dual gating by eATP and regulated expression of P2X7 can imprint distinct outcomes to the T cell depending on the metabolic fitness and/or developmental stage. In the thymus, signaling by P2X7 contributes to γδ T cell lineage choice. In secondary lymphoid organs, P2X7 stimulation promotes Th1/Th17 polarization of $CD4^+$ naïve cells, Tregs conversion to Th17 cells and cell death of Tfh cells that are not stimulated by cognate antigen. Moreover, P2X7 stimulation in eATP rich microenvironments, such as damaged and/or inflamed tissues as well as tumors, induces cell death of various T cell effector subsets.

Keywords: P2X7, T cell, extracellular ATP (eATP), T cell effector function, mucosal immunology, T cell development

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INTRODUCTION

Signaling by adenosine triphosphate (ATP) emerged very early in evolution and is involved in the regulation of highly diverse biologic functions. Trimeric ATP-gated ionotropic P2X receptors are amongst the most ancient signaling channels, having been present in single-cell protozoa and algae (1). The first evidence of T cell responsiveness to extracellular ATP (eATP) dates back to 1989, when Di Virgilio et al. showed that eATP induced plasma membrane depolarization and permeability to low MW dyes, possibly leading to cell death (2). It was then hypothesized that endogenously generated eATP promoted the effector function of cytotoxic T cells via purinergic receptors (3). Subsequent experiments indicated that activation of P2X receptors in T cells could contribute to the outcome of TCR stimulation both in murine and human cells (4, 5). As in other cells of the immune system, the P2X7 receptor subtype stands out among P2X family members as the most important regulator of T cell function. It is a non-selective cationic channel characterized by dual gating: receptor exposure to low concentrations of ATP (e.g., micromolar range) results in small-amplitude currents, whereas stimulation with ATP in the hundreds micromolar range leads to opening of a cytolytic pore and cell death (6). Cryoelectron microscopy of the rat receptor in apo (closed pore) and ATP-bound (open pore) states has unraveled structural insights into P2X7 architecture, which confer the functional peculiarities that distinguish it from the other P2X family members, namely low affinity for ATP, lack of desensitization and cell death initiation (7). In particular, P2X7 combines a P2X domain with a unique "C-cysteine anchor" intra-cytoplasmic motif and a C-terminal cytoplasmic ballast domain (which contains a Zn coordinating cysteine motif and a GDP-binding region), both of which are not present in other P2X receptors. The C-terminal region of P2X7 has been recently hypothesized to originate from the capture of a ballast domain by a P2X gene in ancestral jawed vertebrates (8).

SIGNALING BY P2X7

The human and mouse genes encoding for P2X7 are located in syntenic regions of chromosome 12 and 5, respectively, in close proximity with the gene encoding the P2X4 receptor. Numerous splice variants have been identified for the P2X7 receptor in different species, however, the functional characterization of the various protein isoforms is largely incomplete [reviewed in (9)]. The *P2RX7* gene is highly polymorphic and single nucleotide polymorphisms (SNPs) can significantly influence the functional properties of the receptor (10). Genetic association studies support non-synonymous SNPs (NS-SNPs) in the *P2RX7* gene as an important genetic factor that alters the susceptibility of individuals to various pathological conditions. The predominant expression of P2X7 in cells of the immune system correlates with detection of NS-SNPs in diseases, in which immune system cells play a pivotal role in the pathogenesis [reviewed in (11)].

In addition to eATP, non-nucleotide agonists, including cathelicidins, amyloidogenic peptide β, and serum amyloid, have been suggested to activate P2X7 or act as positive allosteric effectors (10). Moreover, the murine P2X7 receptor can be ADP-ribosylated by the ADP-ribosyltransferase 2.2 (ART2.2) that catalyzes the transfer of ribose from nicotinamide adenine dinucleotide (NAD^+) to R125 in the ectodomain of the P2X7 receptor, resulting in its activation (12). In T cells, P2X7 activation by ADP-ribosylation causes calcium flux, phosphatidylserine exposure, shedding of L-selectin (CD62L), cell shrinkage, pore formation and propidium iodide uptake (13). This alternate mechanism of P2X7 activation is not observed in humans, which lack ART2.1 and ART2.2 (14), and is particularly relevant in murine T cells compared to other cells because of the specific expression of a P2X7 splice variant, that is sensitive to activation by ADP-ribosylation (15–17). The high sensitivity of immunosuppressive T regulatory cells (Tregs) to depletion by NAD^+ released during cell damage or inflammation led to hypothesize a function for the ART2-P2X7 pathway in murine Tregs homeostasis (18). An important consequence of P2X7 gating by ADP-ribosylation is the "spontaneous" P2X7 activation of T cells (19) and reduced vitality of Tregs, tissue-resident memory (Trm) (20) and natural killer T cells (21) that co-express high levels of ART2.2 and P2X7, during the isolation procedure from mice. This phenomenon has been successfully counteracted by the injection of ART2.2-blocking nanobodies prior to organ harvesting (20, 22). The shedding of CD62L mentioned above as well as of CD27 and IL-6 receptor (IL-6R) by P2X7 stimulation, are due to P2X7-mediated activation of metalloproteases, such as ADAM10 and ADAM17 (23–25). Since CD62L promotes T cell homing to secondary lymphoid organs (SLOs), P2X7 activation in naïve T cells stimulated by cognate antigen might promote their egress from SLOs. Interestingly, Tregs expressing the ATP-degrading enzyme ectonucleoside triphosphate diphosphohydrolase-1 (CD39) ameliorated contact hypersensitivity reactions by suppressing ATP-induced CD62L shedding and promoting $CD8^+$ cells retention in skin-draining lymph nodes (LNs) (26). Another possible important target of P2X7 induced metalloprotease activation in T cells is CD27, a member of the tumor necrosis factor receptor family, which supports antigen-specific expansion and T cell memory generation (27, 28). Since CD27 activation by interaction with its ligand CD70 is crucial for the outcome of T cell response (29), P2X7-mediated shedding of CD27 might contribute to the regulation of adaptive immunity and/or immunopathology. Along another line, the induction of IL-6R shedding by P2X7 could condition T cell polarization toward pro-inflammatory vs. immunosuppressive programs. These observations indicate the pleiotropic role this P2X7 feature might have in conditioning T cell function.

P2X7 IN T CELL DEVELOPMENT

αβ and γδ T cell development in the thymus is characterized by transition of thymocytes through multiple checkpoints, most of which are regulated by the rearrangement status and specificity of the clonotypic TCR. Whereas, γδ cells develop from $CD4^-8^-$ double negative (DN) thymocytes, αβ cells progress from DN to mature MHCI and MHCII restricted $CD8^+$ and $CD4^+$ T cells, respectively, through an intermediate $CD4^+8^+$ double positive (DP) stage, in which TCR specificity dictates either positive or negative selection of cells (30). The analysis of the dynamics of changes in cytosolic Ca^{2+} elicited by eATP in thymocytes via P2X7 receptor showed significant variations between individual cells that were dependent on the developmental stage. It was hypothesized that eATP could promote differentiation of most immature DN cells in the outer cortex; conversely, progression to the DP stage in the inner cortex would correspond to loss of responsiveness to eATP via P2X7, thus protecting positively selected cells from eATP released during massive apoptosis of neglected or negatively selected DP cells (31). More recently, this phenomenon was explained by the demonstration of the direct binding of histone deacetylase (HDAC) 3 to the *P2rx7* enhancer and repression of P2X7 signaling in DP cells (32). Nevertheless, protection of DP cells from death by pharmacological P2X antagonism could suggest some function of P2X7 in the elimination of neglected DP cells [(33); **Figure 1A**].

TCR signal strength is a crucial determinant in T cell fate. Increased signal strength of γδTCR with respect to pre-TCR results in induction of the γδ differentiation program. P2X7 signaling contributes to γδ lineage choice by promoting ERK phosphorylation and induction of early growth response (*Egr*) transcripts. Moreover, the impairment of the ERK-Egr-inhibitor of differentiation 3 (Id3) signaling pathway in γδ cells from $P2rx7^{-/-}$ mice resulted in diversion of γδ T cells to "innate-like" NK1.1-expressing cells with limited TCR diversity (34). These experiments suggest a function of P2X7 in shaping the γδ T cells repertoire, whereas lineage choice and differentiation to mature $CD4^+$ or $CD8^+$ αβ thymocytes do not seem to be affected by P2X7 expression (**Figure 1A**). Whether and how P2X7 activity might influence cell metabolism in conditioning γδ thymocytes differentiation has not been addressed so far.

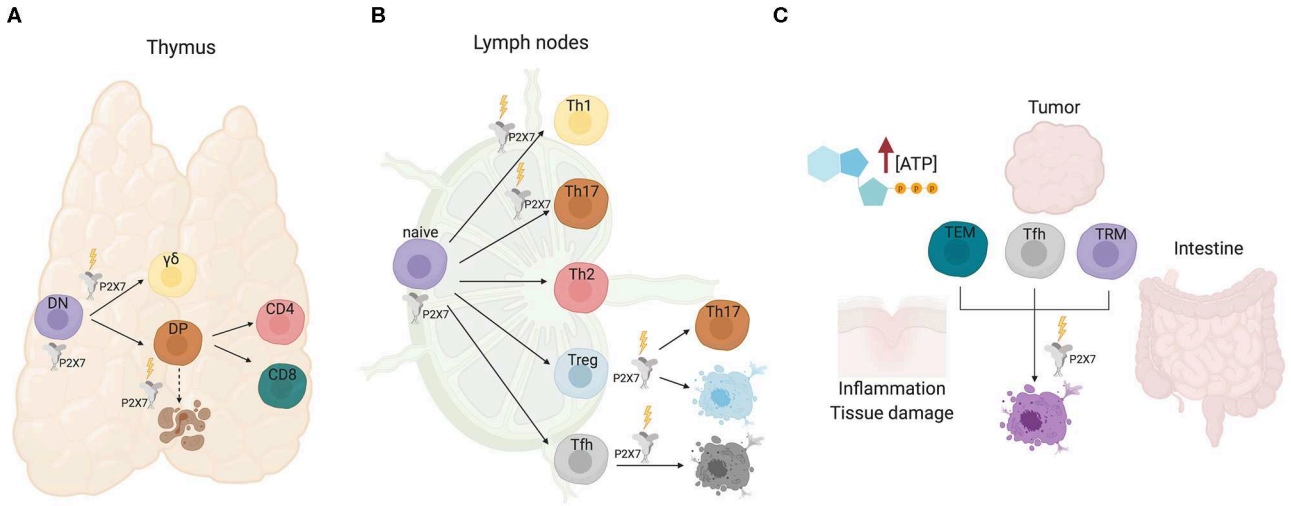

FIGURE 1 | P2X7 activity in T cell development and function. **(A)** In the thymus, P2X7 activity could promote differentiation of immature DN cells. Signaling by P2X7 contributes to γδ lineage choice by promoting ERK phosphorylation and induction of early growth response (*Egr*) transcripts. Conversely, progression to the DP and single positive CD4 or CD8 stages is not influenced by P2X7, albeit P2X7 might function in cell death induction of neglected DP cells. **(B)** In secondary lymphoid organs, P2X7 stimulation promotes Th1/Th17 polarization of $CD4^+$ naïve cells, Tregs conversion to Th17 cells and cell death of Tfh cells that are not stimulated by cognate antigen. **(C)** Sensitivity of TEM, Tfh, and tissue resident memory T cells to P2X7 mediated cell death in eATP rich microenvironments.

P2X7 IN NAÏVE T CELL RESPONSE

In T cells, the increase in the concentration of cytosolic Ca^{2+} that follows TCR stimulation by peptide/MHC complex is accompanied by mitochondrial uptake of Ca^{2+}. This phenomenon avoids cellular Ca^{2+} overload, and contribute to a rapid clearing of Ca^{2+} in spatially restricted areas, such as near Ca^{2+} channels in the plasma membrane or the ER (35). Moreover, mitochondrial uptake of Ca^{2+} stimulates the aerobic synthesis of ATP (36, 37). TCR triggering of naïve T cells results in ATP release via pannexin-1 hemichannels and autocrine stimulation of P2X receptors in the plasma membrane. Murine naïve CD4 T cells express *P2rx1*, *P2rx4* transcripts, and higher levels of *P2rx7*. The ATP released upon naïve T cell activation functions as an autocrine stimulus and sustains MAPK signaling and induction of pro-inflammatory programs via P2X receptors stimulation (**Figure 1B**). Accordingly, pharmacological antagonism of P2X activity promoted T cell anergy and showed beneficial effects in autoimmune conditions (38). These effects were also favored by the conversion of naïve CD4 T cells into immunosuppressive T regulatory cells (Tregs) (39). Autocrine signaling by eATP via P2X7 receptor was shown to contribute to TCR-mediated Ca^{2+} influx, NFAT activation and IL-2 production in human CD4 T cells; blocking of P2X7 signaling inhibited T cell activation, suggesting P2X7 receptor is required for effective T cell activation (40). Importantly, expression of CD39 and CD73, the ecto-5′-nucleotidase that degrades extracellular AMP into adenosine, by other immune and tissue resident cells can dramatically condition the outcome of T cell responses (41–43). The *P2xr7* gene is robustly upregulated in T effector/memory (TEM) cells. P2X7 activity seems to play different functions in regulating the proliferative response of naïve vs. TEM cells upon TCR stimulation. Murine *P2rx7*$^{-/-}$ CD4 naïve cells did not show any difference in cell proliferation as compared to WT cells upon TCR stimulation, suggesting that P2X1 and/or P2X4 could compensate for the lack of P2X7 activity, an observation made also in human T cells (44). In contrast, stimulation of *P2rx7*$^{-/-}$ TEM cells revealed a peculiar enhancement of cell cycling activity with respect to the WT counterpart (our unpublished observations). This phenomenon could be due to the sustained generation of mitochondrial reactive oxygen species (ROS) that was associated to P2X7 activity in T cells (45), and induction of premature cellular senescence.

P2X7 ACTIVITY IN EFFECTOR/MEMORY T CELL FUNCTION

Extracellular ATP is virtually absent in the interstitium of tissues in physiological conditions with the notable exception of the intestine, where eATP generated by the microbiota can permeate enterocytes (46). In contrast, damaged and/or inflamed tissues as well as tumors' microenvironment (TME) are characterized by eATP concentrations that can reach the millimolar range (47–49). Therefore, P2X7 expression can crucially impact the outcome of local immune system response. In this respect, we have shown that P2X7 stimulation in immunosuppressive T regulatory cells (Tregs) can result in conversion into pro-inflammatory IL-17 secreting cells, thereby possibly worsening the inflammatory tissue damage in pathological conditions [(39); **Figure 1B**]. Analogously, P2X7 receptor inhibition promoted long-term cardiac transplant survival in murine recipients of fully

mismatched allograft by reducing T cell activation and Th1/Th17 differentiation (50).

In T follicular helper (Tfh) cells, conversely, P2X7 stimulation restricts the expansion of aberrant cells and the generation of self-reactive antibodies in experimental murine lupus, but its activity is dispensable for regulation of antigen-specific Tfh cells during parenteral vaccination. P2X7 stimulation likely controls the development of pathogenic $ICOS^+$ IFN-γ-secreting Tfh cells, which characterize systemic lupus erythematosus (SLE), by inducing pyroptosis via caspase-mediated activation of gasdermin D (**Figure 1B**). Notably, SLE patients are characterized by reduced P2X7 activity in circulating Tfh cells (51). Acute TCR stimulation of Tfh cells robustly downregulates *P2rx7* expression, thus protecting antigen responding T cell from cell death (52). Similar results have been obtained in tissue resident memory T cells, suggesting that selective downregulation of *P2rx7* in T cells that productively respond to cognate antigen would ensure the amplification of pathogen-destructing cells during infections (53). In contrast, P2X7 activity is required for the establishment and maintenance of long-lived central and tissue-resident memory CD8 T cells in mice, probably reflecting the function of P2X7 as ion channel in promoting mitochondrial function and metabolic fitness (54).

P2X7-MEDIATED T CELL CONDITIONING IN THE INTESTINE

The intestinal microbiota influences host physiology, metabolism, and immune system homeostasis. The interaction between microbes and mammalian immune system results in the selection and "tolerance" of beneficial species. Within this inter-kingdom relationship, eATP plays an important role as a released bacterial metabolite capable of modulating immune system function. The first evidence that commensal bacteria-derived ATP could condition host immune system was provided by Atarashi et al. by showing that a $CD70^{high}CD11c^{low}$ subset of lamina propria cells could be activated by intestinal ATP and induce the differentiation of pro-inflammatory Th17 cells (55). Extracellular ATP was shown to activate dendritic cells (DCs) via P2X7, thereby polarizing the T cell response in a number of physiological and pathophysiological conditions (48, 49, 56–60). However, whether P2X7 stimulation in DCs was responsible for the induction of Th17 cells by intestinal microbiota-derived eATP was not established. Signaling by P2X7 is responsible for cell death of Tfh cells in the Peyer's patches of the small intestine by bacteria-derived ATP, a mechanism important in ensuring controlled generation of T cell dependent secretory IgA (52) and a beneficial shaping of gut microbiota composition (61). The intestinal microenvironment profoundly influences the sensitivity of intraepithelial CD8 cells, both the CD8αβ and CD8αα expressing subset, to P2X7 mediated cell death. In fact, retinoic acid causes up-regulation of P2X7 on purified CD8 T cells and induces responsiveness to extracellular nucleotides. Accordingly, lack of P2X7 led to enhanced $CD8^+$ T cell responses in the intestinal mucosa, thus defining P2X7 as a regulatory element in the control of $CD8^+$ T cells in the intestinal mucosa (62). The induction of P2X7 upregulation by retinoic acid was observed also in $CD4^+$ effector T cells. Hashimoto-Hill et al. showed retinoic acid receptor α binding to an intragenic enhancer region of the *P2rx7* gene (63). Probably, this transcriptional control is responsible for the robust expression of P2X7 on most intestinal αβ and γδ T cells, including T-helper type 1 (Th1) and Th17 cells as well as invariant NKT cells (64). Intestinal effector T cells are effectively deleted by P2X7 mediated cell death and P2X7 activation suppressed T-cell-induced colitis in lymphopenic mice (**Figure 1C**). Results obtained with vitamin A-deficient and $P2rx7^{-/-}$ mice indicate that the retinoic acid-P2X7 pathway is important in preventing expansion of aberrantly activated T cells, as observed with "P2X7-hypoactive" Tfh cells in SLE (51). Therefore, it appears that retinoic acid controls intestinal effector T-cell populations by inducing P2X7 expression. This pathway is likely responsible also for P2X7 mediated control of Tfh cells response to oral vaccination, thereby limiting the generation of high-affinity secretory IgA (46).

CONCLUDING REMARKS

Dual gating and regulated expression of P2X7 can imprint distinct outcomes to the T cell depending on the metabolic fitness and/or developmental stage via autocrine signaling or microenvironment's clues, like eATP or other factors (e.g., NAD^+ in mice) conditioning P2X7 activity. The peculiarity of P2X7 function as cationic channel and cytolytic pore could be responsible for some apparently contradictory findings on P2X7 dependent responses in particular T cell subsets in different experimental settings. It would be important to define molecular mechanisms that could affect P2X7 activity in T cells (e.g., gene polymorphism, RNA splicing, microRNAs, long non-coding RNAs) in different physiological and pathophysiological contexts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

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NLRP3 Inflammasome Activation in Cancer: A Double-Edged Sword

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Inflammation is involved in tumor development and progression as well as antitumor response to therapy. In the past decade, the crosstalk between inflammation, immunity, and cancer has been investigated extensively, which led to the identification of several underlying mechanisms and cells involved. The formation of inflammasome complexes leads to the activation of caspase-1, production of interleukin (IL)-1β, and IL-18 and pyroptosis. Multiple studies have shown the involvement of NLRP3 inflammasome in tumorigenesis. Conversely, other reports have indicated a protective role in certain cancers. In this review, we summarize these contradictory roles of NLRP3 inflammasome in cancer, shed the light on oncogenic signaling leading to NLRP3 activation and IL-1β production and outline the current knowledge on therapeutic approaches.

Keywords: NLRP3, inflammasome, cancer, therapeutic targets, activation

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INTRODUCTION

It is well-established that inflammation caused by viral or microbial infections contributes to tumorigenesis. However, emerging evidence have shown that it as well has a pivotal role in most stages of cancer development, besides interfering with the ability of immune system to counteract tumor cells and affecting response to treatment. These mechanisms are mainly driven by innate and adaptive immune cells, such as dendritic cells, macrophages, natural killer (NK) cells, neutrophils, and lymphocytes (1, 2).

One of the central mechanisms contributing to inflammation in immune cells is mediated by special cytoplasmic protein complexes known as inflammasomes. They are divided based on their structural features into nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and absent in melanoma 2 (AIM2)-like receptors (ALRs). In addition, inflammasomes belong to a larger family of receptors known as pattern recognition receptors (PRRs), where their function is the recognition of pathogen- or danger-associated molecular patterns (PAMPs or DAMPs), causing the activation, maturation, and production of pro-inflammatory cytokines (3). Besides, emerging evidence has proposed that inflammasomes act as a "signal integrator" detecting changes in cytoplasmic homeostasis. These perturbations, named as homeostasis-altering molecular processes (HAMPs), are induced by the functional consequences of cellular processes, where the inflammasome responds to a cellular imbalance rather than a molecular pattern, triggering inflammation in a sterile context. This provides hints that inflammasome activation via the HAMP detection pathway might also be involved in disease pathogenesis (4, 5).

Amongst the inflammasomes family, NLRP3 inflammasome is the most characterized. Mutations in NLRP3 are associated with several autoimmune and inflammatory diseases, particularly a group known as cold-induced auto-inflammatory syndrome (CAPS). In addition, NLRP3 has been implicated in several other diseases including inflammatory bowel disease (IBD), rheumatoid arthritis, and Parkinson's disease (6). In cancer, analysis of copy number alterations in tumor samples has shown *NLRP3* with a high frequency of copy gains, thus acting more as an oncogene (7). However, different roles of inflammasomes in tumorigenesis and antitumor immunity have emerged in the past decade (8), without overlooking the well-established role of cytokines in cancer pathogenesis (9).

Here, we discuss the structure and activation pathways of NLRP3, and provide a brief updated review on the most recent research investigating its opposing roles in cancer. Lastly, we list the potential therapeutic targets and the latest reports and clinical trials investigating them.

NLRP3 INFLAMMASOME

Historical Background

Since the cloning of IL-1β in 1984 (10, 11) and the characterization of its various immunological activities, enormous research has been conducted to further explore the biology of cytokines and their effects on inflammation and other physiological roles. The first major contribution following this, was the identification of IL-1-converting enzyme (ICE), now named as caspase-1 (12, 13). Despite that, the underlying mechanisms causing the processing and release of IL-1ß remained unclear. It was only until 2002, when Martinon et al. (14) identified a caspase-activating complex, which leads to the maturation and secretion of IL-1β, now known as the inflammasome. They continued their pioneering work in this field (15), which led to discovering the association of inflammasomes with CAPS (16), as well as gout and type 2 diabetes. Additionally, they reported several inflammasome agonists, PAMPs including muramyl dipeptide (MDP) (17), viral DNA (18) and malaria-associated hemozoin (19); DAMPs such as monosodium urate (MSU) crystals (20); and environment-derived factors like asbestos, silica (21) and alum (22). A number of different clinical trials for inflammasome-related inflammatory diseases were conducted which led to the development of a therapy for CAPS patients in the clinic (23), in addition to promising results in several clinical studies involving gouty arthritis patients treated with anakinra (24, 25). These revolutionary discoveries paved a new path in the fields of inflammasome activation, innate immunity cytokines production, and their involvement in health and disease.

Structure and Activation of the NLRP3 Inflammasome

Inflammasomes are danger-sensing, multimeric protein complexes that are part of the innate immune response. The most widely studied and well-characterized inflammasome is NLRP3, which is characterized by the presence of a central nucleotide-binding and oligomerization (NACHT) domain, which is usually flanked by C-terminal leucine-rich repeat (LRR), and N-terminal pyrin domain (PYD) (**Figure 1A**) (3). In brief, a danger signal sensed leads to a conformational change of NLRP3 causing the exposure of NACHT domain. NLRP3 undergoes oligomerization by homotypic interactions between NACHT domains. As a result, the PYD domain of NLRP3 becomes exposed, recruit the adaptor apoptosis speck protein (ASC, also known as PYCARD) and bind through their shared PYD domains (**Figure 1A**). Following, ASC converts to a prion-like form and generates long ASC filaments. This interaction recruits the CARD of pro-caspase-1 facilitating its binding to the complex. Additionally, the clustering of pro-caspase-1 forms its own prion-like filaments that separates from the ASC filaments allowing the auto-cleavage and formation of the active caspase-1 p10/p20 tetramer, which then processes cytokine pro-forms into active molecules. Therefore, the cluster of oligomerized NLRP3-ASC-pro-caspase-1 complex results in the assembly of the multi-subunit wheel-shaped inflammasome complex (**Figure 1B**) (3, 14, 26–29). The activation of NLRP3 inflammasome causes two main effects, the induction of programmed cell death known as pyroptosis, and/or a pro-inflammatory response caused by the release of inflammatory cytokines IL-1β and IL-18.

The canonical activation process requires two main steps known as priming signal and activating signal (**Figure 1C**). The first step is provided by inflammatory stimuli from toll-like receptors (TLR) ligands or endogenous molecules, which induce the expression of NF-κB. Additionally, other endogenous factors and mechanisms have been identified to prime the inflammasome in sterile inflammatory diseases, such as reactive oxygen species (ROS), hypoxia, metabolites, oxidized low-density lipoprotein (oxLDL), amyloids, and complement. The second step is usually promoted by PAMPs and DAMPs, which cause potassium ion (K^+) efflux, calcium (Ca^{+2}) flux, lysosomal damage or ROS production leading to NLRP3 inflammasome assembly, caspase-1 cleavage, and thus the maturation and secretion of IL-1β and IL-18 (27, 28, 30).

On the other hand, other pathways for NLRP3 inflammasome activation were described (reviewed elsewhere (31, 32). The non-canonical NLRP3 inflammasome pathway is activated by most Gram-negative bacteria, and requires capase-11 (33) as well as vacuolar rupture mediated by interferon-inducible guanylate-binding proteins (GBPs). Also, an alternative NLRP3 inflammasome pathway is activated in human monocytes induced by LPS and requires the molecules RIPK1, FAS-associated death domain protein (FADD), and caspase-8 (34).

NLRP3 Inflammasome in Cancer

The function of NLRP3 inflammasome in human cancers is rather a conflicting topic (8, 35), where there is evidence of a protective anti-tumorigenic effect as well as a pro-tumorigenic role in different types of cancer (summarized in **Table 1**). Here, we discuss both roles shown in murine and human studies and introduce new insights for the effect of oncogenic mutations in inducing NLRP3 inflammasome activation in leukemias.

NLRP3 inflammasome have been shown to promote the development of several cancers, where most studies were

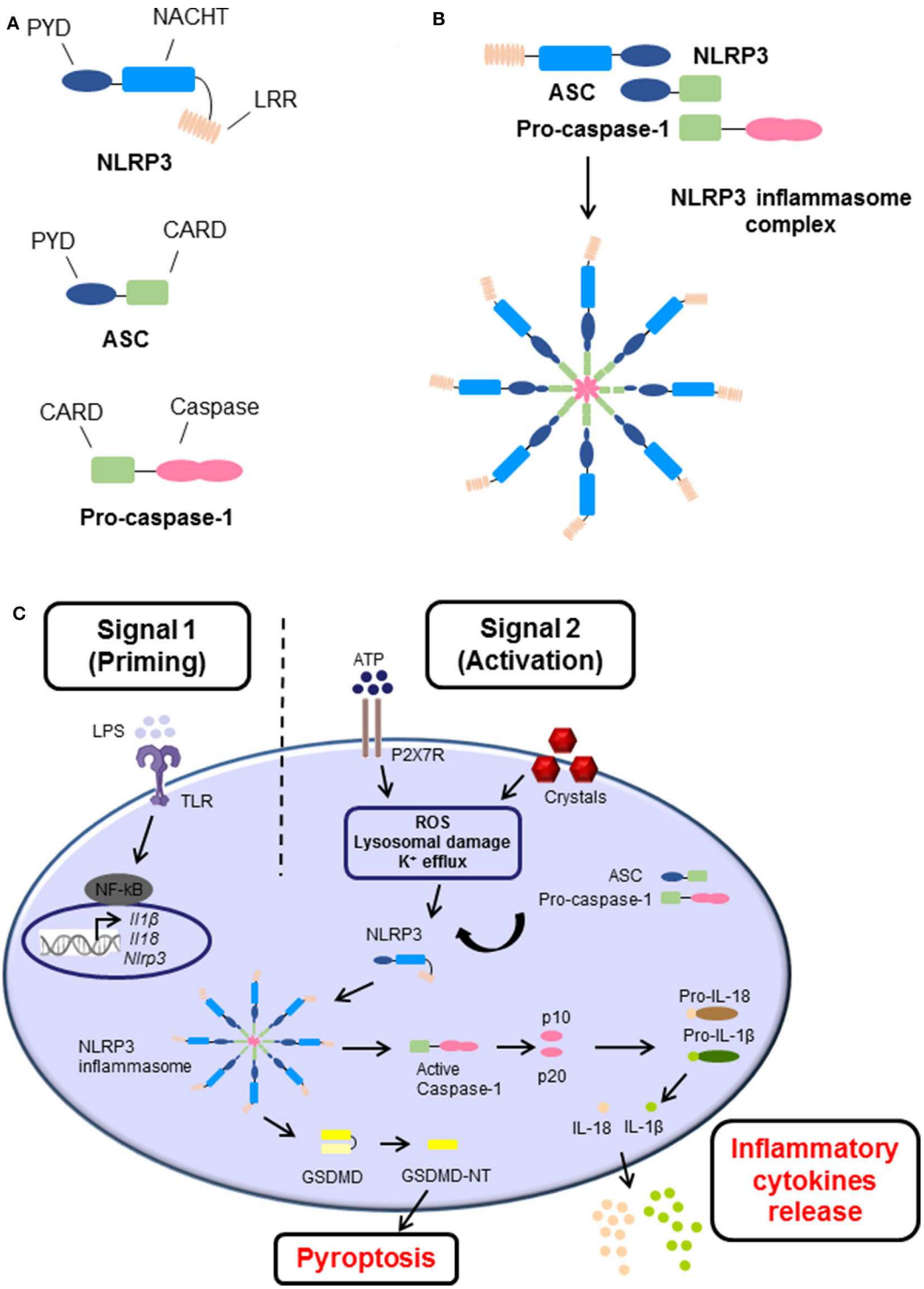

FIGURE 1 | The structure and canonical activation of the NLRP3 inflammasome complex. **(A)** The structure of NLRP3 is comprised of three main domains: (i) NLRP3, containing an N-terminal pyrin domain (PYD), a central NACHT domain, and a C-terminal leucine-rich repeat (LRR) domain; (ii) adaptor apoptosis speck (ASC) which contains PYD and CARD domains; and (iii) pro-caspase-1 which contains caspase-1 and CARD domains. **(B)** Upon activation, NLRP3 undergoes oligomerization, recruits, and binds ASC, which subsequently recruits and binds pro-caspase-1 via their shared domains. The formation of this NLRP3 inflammasome cluster results in a prion-like assembly of the complex. **(C)** The activation process of NLRP3 inflammasome consists of two main signals: (i) Signal 1 (Priming), which is induced by pathogen recognition receptors (PRRs) such as toll-like receptors (TLRs) activated by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), or other endogenous factors and mechanisms such as reactive oxygen species (ROS), hypoxia, metabolites, oxidized low-density lipoprotein (oxLDL),

(Continued)

FIGURE 1 | amyloids, and complement (not shown). This leads to the transcriptional upregulation of *Nlrp3*, *Il1b*, and *Il18* via transcription factors such as NF-κB. (ii) Signal 2 (Activation), is provided by PAMPs or damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP) and crystals which activate different signaling events including ROS, lysosomal damage and K^+ efflux, leading to activation and recruitment of NLRP3, oligomerization, and formation of NLRP3 inflammasome complex. The activation and formation of NLRP3 inflammasome has two main consequences: (i) cleavage of Gasdermin D GSDMD and inducing pyroptosis and/or (ii) auto-cleavage and formation of the active caspase-1 and p10/p20 tetramer which then proteolytically cleaves pro-IL-1β and pro-IL-18 into their bioactive forms IL-1β and IL-18 prior to their release.

focused on proliferation, survival, metastasis, angiogenesis, and immunosuppression. In breast cancer, NLRP3 inflammasome, and IL-1β production promote the infiltration of myeloid cells such as myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), providing an inflammatory microenvironment thus promoting breast cancer progression (36). In addition, NLRP3 inflammasome in fibroblasts is further linked with progression and metastasis (37), and IL-1β was found to have an immunosuppressive, pro-tumorigenic in the tumor microenvironment (38). Besides, the NLRP3 inflammasome seems to be an effector for promoting metastasis via the lymphatic system and favoring mammary carcinoma development (39). Interestingly, Huber et al. (42) demonstrated that IL-18 induced by NLRP3 causes the downregulation of the soluble IL-22 receptor, IL-22-binding protein (IL-22BP), leading to an increase in the ratio of IL-22/IL-22BP, which at later stages promotes tumor development (42). Additionally, NLRP3 deficiency leads to suppression of metastases and methylcholanthrene (MCA)-induced sarcomas in mouse models, which were dependent on NK cell and IFN-γ (44). In epithelial skin cancer, mice deficient for IL-1R and caspase-1 showed partial protection against skin cancer development (43). Besides, the roles of inflammasomes in melanoma pathogenesis is established (65). In particular, NLRP3 inflammasome was shown to be constitutively expressed and activated in human melanoma cells. However, these cells secrete biologically active IL-1β in an autonomous way without the presence of exogenous stimuli at late stages of the disease (54). In HNSCC, NLRP3 inflammasome is found upregulated in carcinoma tissues and associated with carcinogenesis and cancer stem cells (CSCs) self-renewal activation (46–48). Also, NLRP3 signaling seems to drive immunosuppression in pancreatic carcinoma, by promoting tolerogenic T cell differentiation and adaptive immune suppression via IL-10 (56).

In hematological malignancies, the role of NLRP3 inflammasome in normal and malignant hematopoiesis has been lately reviewed (66). We have recently reported a novel function of the NLRP3 inflammasome in the pathogenesis of hematological malignancies, particularly myeloproliferation in leukemias. Interestingly, and despite the manifestation of oncogenic *KRAS* in hematopoietic cells, we could show that the NLRP3 inflammasome has a key role in the development of several myeloid leukemias features *in vivo*, including cytopenias, splenomegaly, and myeloproliferation. These phenotypes are often seen in chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML) and more rarely acute myeloid leukemia (AML) patients harboring *KRAS* mutations. Additionally, we found evidence of NLRP3 inflammasome activation upon analyzing JMML, CMML, and AML patient samples harboring *KRAS* mutations, providing a stronger evidence of the participation of NLRP3 inflammasome in the disease development (49). An open question remains how the NLRP3 inflammasome activation drives hematological malignancies, whether by a cell-autonomous signal that promotes cell proliferation directly or via a modification of the TME or both.

Conversely, NLRP3 inflammasome was also shown to have an anti-tumorigenic role. Previously, Ghiringhelli et al. (67) have proposed that NLRP3 inflammasome is required for dendritic cell-mediated priming of IFN-γ-producing T lymphocytes against tumor cells. NLRP3 inflammasome seems to act as a negative modulator of tumorigenesis in colitis-associated cancer (59), which is confirmatory to the study emphasizing the role of NLRP3 inflammasome in the regulation of intestinal homeostasis and thus protection against colitis (60). In addition, NLRP3 inflammasome deficiency seems to cause increased tumor burdens in colorectal cancer. Moreover, Dupaul-Chicoine et al. (62) reported that NLRP3 inflammasome-mediated IL-18 production suppresses colorectal cancer metastatic growth in the liver. In contrast to the tumor-promoting function of IL-22 discussed above, NLRP3/IL-18-mediated downregulation of IL-22BP under controlled production can also provide protective roles against intestinal tissue damage during the inflammation peak (42). In melanoma, it was shown that NLRP3 in the TME weakens the anti-tumor immune response to a cancer vaccine, by assisting the migration of myeloid-derived suppressor cells (MDSCs), thus suppressing the T cell response (64).

NLRP3 inflammasome signaling in humans is controlled by a variety of factors, such as genetic polymorphisms and mutations that can affect gene expression and ultimately lead to its activation. These effects were seen in patients with inflammatory diseases (68–71). Similarly, genetic polymorphisms involved with NLRP3 inflammasome have also been linked to cancer. For instance, a single-nucleotide polymorphism (SNP) in the *NLRP3* gene, *Q705K* (rs35829419), was correlated with poorer survival in patients with invasive colorectal cancer (41), postulated as a risk allele for sporadic metastatic melanoma in Swedish males (72), and also occurs at high frequency in pancreatic cancer patients (73). Additionally, those with NLRP3 polymorphisms (rs10754558 and rs4612666) are more susceptible to gastric cancer when infected with *Helicobacter pylori* (74). In hematological malignancies, polymorphisms restricted only to *IL-1β* and *IL-18* were associated with clinical and pathophysiological characteristics in AML and chronic myeloid leukemia (CML) (75, 76). Besides, studies utilizing gene expression profiling have also implicated the upregulation of NLRP3 inflammasome in several cancers. For example, NLRP3 is overexpressed in HNSCC, LSCC, and squamous cell carcinoma

TABLE 1 | The dual effect of NLRP3 inflammasome in cancers.

Type of cancer	Role and mechanism of action	References
Pro-tumorigenic role		
Breast cancer	NLRP3 and IL-1β promoted tumor growth and metastasis via infiltration of myeloid cells (MDSCs and TAMs) providing an inflammatory microenvironment	(36)
	Murine and human cancer-associated fibroblasts sense DAMPs and activate NLRP3 inflammasome pathway leading to IL-1β secretion	(37)
	IL-1β in a TNBC mouse model has an immunosuppressive, pro-tumorigenic role in the TME, and blocking it improves checkpoint inhibition by anti-PD1	(38)
	S1PR1 on TAMs is associated with NLRP3 expression and correlated with lymphangiogenesis and metastasis	(39)
Colon cancer	NLRP3 is highly expressed in mesenchymal-like colon cancer cells (SW620). NLRP3 is upregulated in colon cancer epithelial cells HCT116 and HT29 during EMT via TNF-α and TGF-β1	(40)
Colorectal cancer	NLRP3 polymorphisms are correlated with poorer survival in patients with invasive CRC patients	(41)
	NLRP3 senses tissue damage, promotes IL-18 which downregulates IL-22BP leading to IL-22 production and promoting tumor development at later stages	(42)
Epithelial skin cancer	IL-1 and caspase-1 play a role in tumor development. ASC expressed in infiltrating myeloid cells acts as a driver of tumorigenesis	(43)
Fibrosarcoma	NLRP3 acts as a suppressor of NK cell antimetastatic function and $CD11b^{+}Gr\text{-}1^{intermediate}$ ($Gr\text{-}1^{int}$) myeloid cells causing decreased levels of CCL5 and CXCL9	(44)
Gastric cancer (GC)	NLRP3 inflammasome activation and IL-1β secretion is upregulated in GC, induce epithelial cells proliferation and tumorigenesis by binding to cyclin-D1 promoter which could be reversed by miRNA-22	(45)
HNSCC	P2X7 and NLRP3 is upregulated in carcinoma tissues and had a role in survival and invasiveness of HNSCC	(46)
	NLRP3 is associated with inflammation-induced carcinogenesis and CSCs markers	(47)
	NLRP3 is overexpressed in human HNSCC tissues, and IL-1β levels were increased in their peripheral blood	(48)
Leukemias (CMML, JMML, and AML)	NLRP3/IL-1β cause myeloproliferation and cytopenias in KRAS-mutant leukemias, mediated by RAC1 activation and ROS production	(49)
LSCC	NLRP3 expression is higher in human cancer tissues compared to normal tissues. High expression of NLRP3 and IL-1β is correlated with a poorer prognosis	(50)
Lung cancer	NLRP3 inflammasome activation enhances the proliferation and metastasis of lung adenocarcinoma cell line A549, mediated by AKT, ERK1/2, CREB, and upregulation of SNAIL	(51)
Lymphoma	NLRP3 inflammasome, through IL-18, promotes lymphoma cell proliferation and inhibits apoptosis, via upregulation of C-MYC, BCL2, and downregulation of TP53 and BAX	(52)
Melanoma	Inhibition of NLRP3 by thymoquinone suppresses metastasis of murine and human melanoma cells by deregulation of IL-1β and IL-18	(53)
	NLRP3 is activated in human melanoma cells, but also constitutively secrete IL-1β via NLRP3 and IL-1R in the absence of exogenous stimulation	(54)
Myelodysplastic syndromes (MDS)	NLRP3 inflammasome is overexpressed in MDS HSPCs, drives clonal expansion and pyroptosis via alarmin signals, gene mutations, and ROS production.	(55)
Pancreatic ductal adenocarcinoma	NLRP3 promotes differentiation of $CD4^{+}$ T cells into tumor promoting Th2 cell, Th17, and regulatory T cell population and suppresses cytotoxic $CD8^{+}$ T cell, mediated by IL-10	(56)
Prostate cancer	Hypoxia causes priming of NLRP3 and AIM2 through upregulation of their receptors and pro-IL-1β	(57, 58)
Anti-tumorigenic role		
Colitis-associated cancer (CAC)	NLRP3, PYCARD, or caspase-1 deficiency causes worse disease outcome and morbidity via increased IL-1β and IL-18 secretion	(59)
	NLRP3 or ASC and caspase-1 deficiency leads to higher susceptibility to DSS-induced colitis and mortality rate due to decreased IL-18 levels	(60)
Colorectal cancer (CRC)	Lack of NLRP3 or caspase-1 causes reduced tumor burden due to decreased levels of IL-18 and impaired production and activation of IFN-γ and STAT1	(61)
	NLRP3 inhibits CRC metastatic growth in the liver by IL-18, NK cells, and increased expression of FasL	(62)
	NLRP3 senses tissue damage, promotes IL-18 which downregulates IL-22BP leading to IL-22 production and exerting protective effects against intestinal tissue damage at the peak of inflammation	(42)
Hepatocellular carcinoma (HCC)	NLRP3 inflammasome components were absent or significantly downregulated in human HCC. NLRP3 deficiency is correlated with advanced stages	(63)
Melanoma	NLRP3 inflammasome impairs anti-tumor response by facilitating migration of myeloid-derived suppressor cells (MDSCs)	(64)

AML: acute myeloid leukemia, CMML: chronic myelomonocytic leukemia, CSCs: cancer stem cells, DAMPs: Danger associated molecular patterns, DSS: dextran sodium sulfate, EMT: epithelial-mesenchymal transition, HNSCC: Head and neck squamous cell carcinoma, HSPCs: hematopoietic stem and progenitor cells, IL-22BP: IL-22- binding protein, JMML: juvenile myelomonocytic leukemia, LSCC: laryngeal squamous cell carcinoma, NK: natural killer, PDAC: Pancreatic ductal adenocarcinoma, ROS: reactive oxygen species, S1PR1: S1P receptor 1, TAMs: Tumor associated macrophages, TGF-β1: transforming growth factor-β1, Th2: T helper type 2 cell, TME: tumor microenvironment, TNF-α: tumor necrosis factors-α, Triple Negative Breast Cancer (TNBC).

tissues compared to normal tissues, and often correlated with poor prognosis and worse pathology (48, 50, 77). In bladder cancer, high expression of NLRP3 inflammasome is also found, making it a potential biomarker for its diagnosis (78). Further studies will be required to understand the association between genetic polymorphisms or differential expression of NLRP3 inflammasome and clinical features of cancer.

The understanding of this crosstalk between immunity, inflammasomes, inflammation, and cancer is the foundation for implementing anti-inflammatory therapeutic options in cancer prevention and treatment.

THERAPEUTIC POTENTIAL OF TARGETING NLRP3 INFLAMMASOME IN CANCER

The involvement of the NLRP3 inflammasome in several inflammation-related diseases, including cancer, provided it as an attractive potential target in designing new drugs for treatment. Several reported molecules and drugs were shown to regulate the inflammasome activity. However, many indirectly affect the inflammasome effector functions by targeting other molecules. Until today, current treatment of NLRP3 inflammasome-related diseases in the clinic involve targeting IL-1β or IL-1β receptor by monoclonal IL-1β antibodies or recombinant IL-1 receptor antagonists. Nevertheless, several specific small-molecule compounds have been shown to have anti-inflammatory effects. Here, we review the variety of NLRP3 inflammasome inhibitors which either target components of its canonical signaling pathway or are specific to NLRP3 protein (summarized in **Table 2**).

Anakinra is a recombinant form of interleukin-1 receptor antagonist (IL-1Ra) (79), which was approved by the US Food and Drug Administration (FDA) for the treatment of rheumatoid arthritis patients and autoinflammatory disorders (122, 123). We have recently reported that treating $Kras^{G12D}$-mutant leukemia mouse models with anakinra improves myeloproliferation and cytopenia phenotypes (49). Due to its clinical safety record and short life, anakinra is an ideal drug to be used in conjugation with chemotherapy. Indeed, one clinical trial on metastatic colorectal cancer reported that the treatment of anakinra besides fluorouracil (5-FU) plus bevacizumab showed survival benefit (80), while another showed improved outcome in PDAC patients when combining anakinra with gemcitabine, nab-paclitaxel, and cisplatin (AGAP) (81). Although older reports indicated that anakinra alone was not able to induce myeloma cell death, a study involving multiple myeloma patients used anakinra in combination with low-dose weekly dexamethasone, showed an improved survival for over 10 years compared to the controls (82). In breast cancer, the use of pre-clinical mouse models indicated that anakinra treatment decreased tumor growth and bone metastasis (83). Besides, a clinical pilot study investigated the administration of anakinra prior to standard chemotherapy in HER2-negative metastatic breast cancer female patients. The study revealed that 2-weeks of anakinra treatment alone could downregulate the expression of several genes for TLR and IL-1β families, but upregulate the expression of tumor lysis-associated genes like NK and $CD8^+$ T-cells (84). These results indicate a promising outlook for the use of anakinra combined with standard chemotherapy in difference cancers. However, the effectiveness of anakinra in antitumor applications needs further investigation through *in vivo* models and later in clinical trials.

Canakinumab is a human anti–IL-1β monoclonal antibody, known for its high specificity to block IL-1β without interference or cross-reactivity with other IL-1 family members. It was approved by the US FDA and European Medicines Agency for treating CAPS (23, 85). Canakinumab has a half-life of a typical IgG1 antibody (124), which gives it an advantage over recombinant IL-1Ra by ensuring the full inhibition of IL-1β over a lengthier period. Interestingly, Canakinumab Anti-inflammatory Thrombosis Outcomes Study (CANTOS), a randomized, double-blinded clinical trial of 10,061 lung cancer and atherosclerosis patients implemented the use of canakinumab, and resulted in a significant reduction of lung cancer-caused mortality. This antitumor effect was evident in lung adenocarcinoma or poorly differentiated large cell cancer due to the few cases of small-cell lung cancers or squamous cell carcinomas (86). Currently, canakinumab is being applied in clinical trials focusing on non-small cell lung cancer (NSCLC), Triple Negative Breast Cancer (TNBC), colorectal cancer and metastatic melanoma. In particular, two ongoing Phase III clinical trials conducted by Novartis pharmaceuticals (CANOPY-1 and CANOPY-2) are currently investigating pembrolizumab plus chemotherapy with or without canakinumab, or docetaxel with canakinumab in NSCLC (ClinicalTrials.gov Identifier: NCT03626545, NCT03631199). The forthcoming results will provide a better insight on in safety and efficacy of using it as combination treatment. However, investigating the use of canakinumab in other cancers remain less prominent, and relatively requires more recognition.

P2X7R mediates NLRP3 inflammasome activation and cytokine release. However, the role of P2X7R in tumor cells is shown to be either pro-tumorigenic or anti-tumorigenic [reviewed in Savio et al. (87)]. Nevertheless, several reports have evaluated the potential of P2X7R antagonists in different cancers and suggested their efficacy in altering tumor cells and suppressing cancer progression. For instance, inhibition of P2X7R caused attenuated tumor proliferation and invasion in PDAC (88), and decreased invasiveness of A253 cells derived from epidermoid carcinoma (46).

Thalidomide, a sedative or hypnotic drug, was used particularly for morning sickness in pregnant women (100). However, it was shown to have an anti-tumor activity due to its antiangiogenic properties (125, 126), and later be an inhibitor of caspase-1 (127). It has been approved as a first-line therapeutic option in patients with advanced multiple myeloma in combination with other chemotherapy drugs because of its anti-tumor activities, resulting in improved response (101, 102). In prostate cancer, the administration of thalidomide alone or in combination with docetaxel resulted in improved response and overall median survival (103, 104). However, its application in other cancer types, such as metastatic melanoma,

TABLE 2 | A list of compounds targeting NLRP3 inflammasome either indirectly or directly and their therapeutic potential in cancers.

Compound name	Mechanism of action	Reference	Studies in cancer
1. Targets of NLRP3 inflammasome pathway			
NLRP3 inflammasome effectors			
Anakinra	Interleukin-1 receptor inhibitor	(79)	(80–84)
Canakinumab	IL-1β inhibitor	(23, 85)	(86)
NLRP3 inflammasome activators			
P2X7 receptor inhibitors	P2X7R inhibitors	(87)	(46, 88)
NLRP3 inflammasome expression			
Andrographolide	NF-κB inhibitor	(89, 90)	(91–95)
Parthenolide	NF-κB inhibitor	(96)	(97–99)
2. Targets of NLRP3 inflammasome components			
Thalidomide	Caspase-1 inhibitor	(100)	(101–107)
VX-765	Caspase-1 inhibitor	(108)	–
Pralnacasan	Caspase-1 inhibitor	(109)	–
Ac-YVAD-CHO	Caspase-1 inhibitor	(110, 111)	–
3. Direct targets of NLRP3 protein			
MCC950	Directly binds to the Walker B motif of NACHT domain, blocking ATP hydrolysis, and formation of NLRP3 inflammasome	(112, 113)	(48, 49, 55)
Oridonin	NACHT domain and Oridonin share cysteine 279 binding site	(114)	(115, 116)
CY-09	Directly binds NLRP3 motif, leading to the abrogation of ATP binding to NLRP3	(117)	–
OLT1177	Binds to NLRP3 inhibiting its ATPase activity	(118, 119)	–
Tranilast	Directly binds to the NACHT domain of NLRP3 and inhibition of ASC oligomerization	(120, 121)	–

NSCLC and hepatocellular carcinoma (105–107), did not show significant usefulness.

In addition, VX-765 (108), Pralnacasan (109), and Ac-YVAD-CHO (110, 111) are other caspase-1 inhibitors which have shown few but promising results in their potential in NLRP3-related diseases. However, their potential as therapeutic targets in cancer was not investigated.

Other compounds include Andrographolide (89, 90) and Parthenolide (96), which mainly target NF-κB signaling pathway, but the later was also shown to directly inhibit NLRP3 inflammasome by interfering with its ATPase activity (128), have also shown promising results in several cancers (129). For instance, andrographolide was shown to suppress cancer cell proliferation, promote apoptosis in colon cancer (91), breast cancer (92, 93), multiple myeloma (94), and enhance the antitumor effect of 5-FU in colorectal cancer (95). Besides, parthenolide have shown positive results in inhibiting tumor cell proliferation in gastric cancer (97), pancreatic adenocarcinoma (98), colorectal cancer (99). However, these two compounds have not been taken further beyond pre-clinical studies.

A number of small-molecule compounds were proposed to show specific inhibitory effects on NLRP3 activation [reviewed further in detail elsewhere (130, 131)]. One example is MCC950, which prevents NLRP3-induced ASC oligomerization, leading to the inhibition of both canonical and non-canonical NLRP3 inflammasome activation as well as IL-1β secretion, presenting it as a promising agent in NLRP3-related diseases (112). Mechanistic studies have revealed that MCC950 directly binds to the Walker B motif of the NLRP3 central NACHT domain, blocking the hydrolysis of ATP and thus the formation of NLRP3 inflammasome. This action is independent of K^+ efflux, $Ca2^+$ flux, or NLRP3–ASC interactions, and occurs without interfering with TLR signaling or the priming step of NLRP3 activation (112, 113, 130). The use of MCC950 in head and neck squamous cell carcinoma was shown to delay tumorigenesis and improve the antitumor response by reducing the numbers of MDSCs; regulatory T cells (Tregs) and TAMs (48). Besides, MCC950 treatment in MDS was sufficient to halt restore effective hematopoiesis by inhibition of pyroptosis (55). Furthermore, we have recently reported that the use of MCC950 in $Kras^{G12D}$-mutant leukemia mouse models improves myeloproliferation and cytopenia phenotypes, by attenuating NLRP3 inflammasome (49). However, despite its promising potential in Parkinson's disease (132), preclinical and clinical reports studying MCC950 in cancer remain rather limited.

Oridonin is a major bioactive component of herbal plant *Rabdosia rubescens*, and is widely used as an over-the-counter (OTC) herbal medicine for the treatment of inflammatory diseases (130). Studies have shown that Oridonin can specifically inhibit NLRP3 inflammasome activation, where NACHT domain and Oridonin share cysteine 279 binding site (114). The ability of Oridonin to suppress cell proliferation was previously demonstrated in breast (133) ovarian (115) and esophageal (116) cancers. On the other hand, CY-09 (117), OLT1177(118,

119), Tranilast (120, 121) present as promising specific NLRP3 inhibitors. However, their potential in NLRP3-related cancers has not been investigated yet.

In conclusion, despite the promising prospective of the compounds mentioned above, further studies are still needed to fully understand their therapeutic potential in NLRP3-related diseases, especially in cancers.

SUMMARY

Despite the well-characterized crucial functions for NLRP3 inflammasome in the immune system, their roles in cancer remain rather complicated and elusive. The double-edged sword effect of NLRP3 inflammasome in cancer appears to be dependent on several factors, including its levels of expression, downstream effector molecules (i.e., IL-1β or IL-18), cancer type, stages of tumorigenesis as well as the potential presence of mutations affecting NLRP3 expression. Therefore, in order to further understand these roles, future research needs to address several points: (i) driving factors of NLRP3 inflammasome activation in tumors, such as oncogenic mutations or mutations of inflammasome components, (ii) possible cross-talk pathways and molecules interacting and affecting the regulation of NLRP3 inflammasome, (iii) effects of TME and its components on NLRP3 inflammasome activation and vice versa, (iv) effect of NLRP3 inflammasome on the regulation of immune cells, antitumor immunity and efficiency of immunotherapy. In summary, targeting the NLRP3 inflammasome or its downstream pathways, either solely or in combination with chemotherapy or other immunotherapeutic approaches, hold a promising potential in cancers.

AUTHOR CONTRIBUTIONS

SH and RZ performed literature research together, discussed the articles, and wrote the manuscript together. All authors contributed to the article and approved the submitted version.

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REVIEW
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Eosinophils and Purinergic Signaling in Health and Disease

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Eosinophils are major effector cells against parasites, fungi, bacteria, and viruses. However, these cells also take part in local and systemic inflammation, which are central to eczema, atopy, rhinitis, asthma, and autoimmune diseases. A role for eosinophils has been also shown in vascular thrombotic disorders and in cancer. Many, if not all, above-mentioned conditions involve the release of intracellular nucleotides (ATP, ADP, UTP, etc.) and nucleosides (adenosine) in the extracellular environment. Simultaneously, eosinophils further release ATP, which in autocrine and paracrine manners, stimulates P2 receptors. Purinergic signaling in eosinophils mediates a variety of responses including CD11b induction, ROI production, release of granule contents and enzymes, as well as cytokines. Exposure to extracellular ATP also modulates the expression of endothelial adhesion molecules, thereby favoring eosinophil extravasation and accumulation. In addition, eosinophils express the immunosuppressive adenosine P1 receptors, which regulate degranulation and migration. However, pro-inflammatory responses induced by extracellular ATP predominate. Due to their important role in innate immunity and tissue damage, pharmacological targeting of nucleotide- and nucleoside-mediated signaling in eosinophils could represent a novel approach to alleviate eosinophilic acute and chronic inflammatory diseases. These innovative approaches might also have salutary effects, particularly in host defense against parasites and in cancer.

Keywords: eosinophils, extracellular ATP, extracellular adenosine, CD39, CD73, P1 receptors, P2 receptors, inflammation

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INTRODUCTION

Nucleotides and nucleosides are present at high concentrations within the cell where they exert multiple functions. However, they are not restricted to the intracellular compartments but they serve as extracellular mediators to eukaryotic cells (1, 2). A growing body of evidence indicates that released nucleotides represent important modulators to several cell and organ pathways under both physiological and pathological conditions. Their role in the cardiocirculatory and the nervous system, in tissue metabolism, respiration and immune function, as well as in gastrointestinal and hepatic disease pathogenesis has been described recently (3–7).

Extracellular purines and pyrimidines have been implicated in the regulation of ciliary beat frequency, chloride/liquid secretion, goblet cell degranulation, epithelial mucus secretion, transmission of the respiratory nervous stimuli and modulation of the airway vascular tone (8–10).

Accordingly, inhaled ATP is a powerful bronchoconstrictor in both healthy and asthmatic individuals. Furthermore, extracellular ATP can act as a damage-associated molecular pattern molecule (DAMP, also known as alarmin or danger molecule) to activate the inflammasome with subsequent upregulation of IL-1β, IL-18, and release of other pro-mobilizing mediators like high molecular group box 1 (Hmgb1) and S100 calcium-binding protein A9 (S100A9) (11).

Under homeostatic conditions extracellular ATP levels are rather low. This is due to a moderate release and rapid degradation by extracellular ATP-metabolizing enzymes (ectonucleotidases) (12, 13). However, in the course of infection, inflammation, hypoxic conditions due to ischemia as well as necrotic and apoptotic cell death ATP is released from intracellular storage pools and can reach a concentration high enough to be sensed by surrounding cells expressing P2 receptors (14–16). Besides the unregulated ATP release as a consequence of cell damage, mediated secretion of this extracellular messenger occurs through plasma membrane molecules such as connexins, pannexins, and P2X7 receptors (17–20). Apart from ATP, uridine nucleotides (UTP, UDP and UDP-glucose) can also be released in the extracellular space (21).

Eosinophils are polymorphonuclear cells mainly involved in the immune defense, tissue remodeling and inflammation. Activation and migration of these cells to inflammatory sites are crucial to tissue defense. In addition to the classical immune activators (chemokines, cytokines, microbial products, allergens, complement components) eosinophils are also capable to sense nucleotides that can amplify responses induced by other stimuli (22). Thus, extracellular nucleotides contribute to eosinophilic inflammation and tissue damage both in human and animal models (23, 24). Therefore, nucleotides and nucleosides are under intense investigation for their capacity to activate and recruit eosinophils. In this regard, high levels of ATP are present in the bronchoalveolar lavage fluid of patients suffering from eosinophilic pneumonia. This mediator also correlates with uric acid and IL-33 concentration (24, 25).

P2 RECEPTORS

P2 receptors are plasma membrane receptors for extracellular nucleotides. On the basis of cloning, functional and pharmacological data, two P2 receptor subfamilies have been described: P2X and P2Y receptors (2, 26). Differences in nucleotide sensitivity and specificity of the P2 receptor subtypes, allow the activation of distinct P2 receptor subsets depending on the nucleotide concentration and kind.

The P2X receptor subfamily represents ligand-gated ion channels selective for monovalent and divalent cations. These ion channels are homo- or in some cases hetero-multimers with carboxyl- and amino-terminal cytoplasmatic domains (27, 28). In mammals, seven different subunits have been identified and named P2X1-P2X7. Extracellular ATP is an agonist for all P2X subtypes and regulates their permeability to Na^+, K^+, Ca^{2+}, Mg^{2+}. While the majority of P2X receptors is rapidly desensitized (e.g., P2X1 and P2X3), the non-desensitizing P2X7 represents a peculiar subtype having a long carboxyl-terminal domain allowing the receptor to undergo a permeability transition from a plasma membrane channel to a large plasma membrane pore depending on ATP concentration and the way of stimulation (26).

Stimulation of the P2X7 subtype by high ATP concentrations is associated with a permeability transition due to the opening of a membrane pore with a cut-off of 900 Da (27). Transmembrane ion fluxes, driven by pore opening, induce transcription and secretion of different inflammatory cytokines such as IL-1β, IL-18, IL-6 (23). Pharmacological blocking, genetic ablation and attenuation of P2X7 function resulted in reduced inflammatory responses (29–31).

The P2Y receptors are seven transmembrane G-protein-coupled receptors with an extracellular amino-terminus and an intracellular carboxyl-terminus. Eight human P2Y subtypes have been identified and named: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (32). They differ in agonist specificity, coupled G-protein and transduced intracellular signaling. However, according to amino acid homology and presence of conserved motifs in the transmembrane α-helix 7, two groups have been described. The first group includes the P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11 subtypes, having 25–52% amino acid identity and a Y-Q/K-X-X-R motif in the transmembrane α-helix 7 (33).

To the second group belong P2Y12, P2Y13 and P2Y14, with sequence homology of 47–48% and the presence of the K-E-X-X-L motif (2). Some evidence suggests that the two P2Y subgroups differ in G-protein coupling. Hence, the receptors of the first group couple to Gq/G11proteins, contributing to calcium release via phospholipase C/inositol-1,4,5-triphosphate activation; while receptors of the second group couple to Gi/0 proteins, inhibiting adenylate cyclase (AC) (34). Different P2Y agonists have been identified, among them both adenine and uridine nucleotides (35). P2Y1, P2Y12, and P2Y13 subtypes are preferentially activated by ADP (36), whereas UDP is an agonist at P2Y6. While P2Y2 can be activated by both UTP and ATP, P2Y4 and P2Y11 are selective for UTP and ATP, respectively. Last but not least, P2Y14 is activated by UDP-glucose (35).

In the last two decades, P2 receptors gained attention for their wide tissue distribution and number of modulated pathophysiological responses. This has also prompted several P2-based therapeutic approaches (37), as in the context of kidney

Abbreviations: AC, adenylate cyclase; ADO, adenosine; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BALF, bronchoalveolar lavage fluid; BzATP, 2',3'-O-(4-benzoyl-benzoyl)ATP; C3a, complement factor 3a; C5a, complement factor 5a; CCL11, (eotaxin-1); CCL24, (eotaxin-2); CCL26, (eotaxin-3); CNS, central nervous system; COPD, chronic obstructive pulmonary disease; CR, complement receptor; DAMP, damage-associated molecular pattern; ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; EPO, eosinophil peroxidase; GM-CSF, granulocyte macrophage-colony stimulating factor; GTP, guanosine triphosphate; HDM, house dust mite; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; LPS, lipopolysaccharides; LT, leukotriene; MBP, major basic protein; MCP, monocyte chemoattractant protein; OVA, ovalbumin; PAF, platelet-activating factor; PGD2, prostaglandin D2; MSCs, mesenchymal stromal cells; PSGL-1, P-selectin Glycoprotein Ligand-1; ROIs, reactive oxygen intermediates; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; UDP, uridine diphosphate; UTP, uridine triphosphate; VCAM-1, vascular cell adhesion molecule 1.

disease, cardiovascular and metabolic disorders as well as central nervous system (CNS) inflammation.

P1 RECEPTORS

Similar to the P2Y receptors, the P1 receptors are seven-transmembrane G-protein-coupled receptors but their natural agonist is adenosine (ADO) (38). ADO exerts ambiguous effects in different tissues, depending on cell type and P1 receptor subtypes predominantly expressed (39–41).

Four receptors subtypes have been identified and named: A1 (ADORA1), A2A (ADORA2A), A2B (ADORA2B), and A3 (ADORA3), respectively. The main differences between the subtypes concern the affinity to ADO, the coupled G-protein families and effects on AC. While A1 and A3 inhibit AC, A2A and A2B drive its activation (38). ADO concentration of the extracellular *milieu* ranges from 100 to 500 nM and increases to levels in the low micromolar range as a consequence of inflammation, hypoxia and ischemia. Among the subtypes, A2B shows the lowest affinity for ADO. Accordingly, A1, A2A and A3 are activated by lower ADO concentrations (10–50 nM); whereas A2B needs a rather high agonist concentration (1 mM) for stimulation.

Primarily, adenosine receptors have been associated with dampening acute inflammation and tissue injury. On the one hand the inhibition of pro-inflammatory cytokine production and on the other hand the induction of suppressive cytokines as well as regulatory immune cell differentiation are two known effects of the anti-inflammatory responses driven by P1 receptors. Nevertheless, in the context of rheumatoid arthritis or multiple sclerosis P1 receptors have also been implicated in inflammatory cell recruitment (42–44).

ECTONUCLEOTIDASES

Four main groups of plasma membrane enzymes are endowed of the ability of hydrolyzing extracellular nucleotides, transforming ATP and ADP to ADO thus shifting purinergic receptor activation from P2 to P1 subtypes. Activity of ectonucleotidases is fundamental to avoid excessive accumulation of nucleotides in the extracellular *milieu* and to terminate P2 signaling (45). The following families have been described: ectonucleoside triphosphate diphosphohydrolases (NTPDases), ecto-5′-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterases (NPP) and alkaline phosphatases (12). NTPDases (among which NTPDase1 or CD39) catalyze the conversion of ATP or ADP to AMP and are highly expressed by immune cells and the vasculature (46–48). Extracellular AMP is further hydrolyzed to the anti-inflammatory ADO by CD73 (49, 50). However, the CD73 driven ADO generation has been associated with the potent suppression of anti-cancer immune responses. Thus, inhibitors of CD73 for the use in clinical practice are highly desired (51).

The proposed important immunoregulatory activities of ectonucleotidases are to prevent the development of autoimmune conditions. Accordingly, we recently observed that CD39 overexpression ameliorates experimental colitis and prevents hypoxia-related damage *in vivo* in a dextran-sulfate-sodium-induced colitis model. In addition, exogenous administration of a recombinant form of human CD39L3 (APT102) boosted the regulatory effects of endogenous CD39 *in vivo* and enhanced *in vitro* Treg functions in Crohn's disease (48). Likewise, the administration of apyrase, which has ectoenzymatic activity comparable to CD39, attenuated peribronchial eosinophilic inflammation and reduced the levels of Th2 cytokines in the bronchoalveolar lavage fluid of mice with allergic airway inflammation (52).

EOSINOPHIL GRANULOCYTES

Eosinophils are granulocytes deriving from $CD34^+$ bone marrow precursors expressing CD38 and CD125. Thereby, IL-3, IL-5, and GM-CSF exposure have been reported to induce eosinophil differentiation (53, 54). The differentiation process occurs in about 8 days and is mainly driven by the transcription factors GATA-1, GATA-2, c/EBP, and XBP1 (55–58). Cytokines (particularly IL-5) and chemokines (CCL11, CCL24, CCL26) promote the release of eosinophils from the bone marrow (59). After circulating in the peripheral blood for 8–12 h, mature eosinophils home into tissues (mammary gland, adipose tissue, uterus, gut, lung) where they contribute to maintaining organ integrity and promote B and T cell immune function (60–64).

Under pathophysiological conditions such as atopic diseases, rhinitis, eczema, asthma and parasitic infections, chemokine-mediated CCR3 receptor activation on eosinophils as well as the stimulation with cytokines such as IL-4, IL-5, IL-9, IL-13, GM-CSF, RANTES, MCP-3, and MCP-4 have been linked to the recruitment and accumulation of eosinophils in tissues including nasal mucosa, lungs, heart, skin, liver and bile ducts, gut and nerves (65–69). In addition, eotaxin, IL-4, and IL-13 have been shown to induce the up-regulation of the adhesion molecules VCAM-1 and PSGL-1 on epithelial cells and fibroblasts thus further promoting eosinophil trafficking and recruitment (70, 71). In contrast, IL-6, and IL-11 decrease tissue infiltration by eosinophils through inhibiting VCAM-1 expression and decreasing production of type 2 cytokines. Pro-inflammatory cytokines such as IL-1, IL-12, and TNF-α up-regulate endothelium adhesion molecules, including VCAM-1, thereby favoring eosinophil diapedesis (67, 72, 73).

Eosinophils themselves produce different cytokines including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, TNF-α, TGF-β, GM-CSF and pro-inflammatory mediators such as leukotriene C4 (LTC4), platelet-activation factor (PAF) (71, 74), the granular cationic proteins, major basic protein (MBP) 1, MBP 2, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). MBP which is present in the crystal core of the specific granules has cytotoxic effects due to interference with electrical properties and permeability of the cell membrane. MBP also triggers degranulation of mast cells and basophils (75). ECP favors the entry of cytotoxic molecules by forming voltage-insensitive, non-selective pores in the membrane of target cells (54, 68). ECP and EDN, that belong

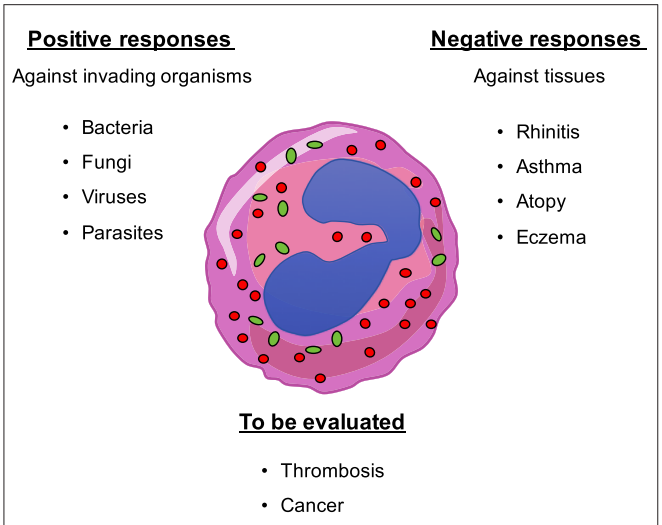

FIGURE 1 | Eosinophils play multiple roles within the organism. Eosinophils are actively involved in the defense against multicellular parasites (e.g., worms) as well as fungi, bacteria, and viruses. However, they also show detrimental responses by damaging tissues and organs such as in rhinitis, asthma, atopy, eczema, etc. Involvement of eosinophils in lung tumor progression and in thrombosis have been also shown but their role has to be adequately evaluated.

to the ribonuclease A superfamily, kill single-stranded RNA pneumoviruses (76). In addition, eosinophils generate reactive oxygen species, hypohalous acids and lysosomal hydrolases that are toxic for bacteria and parasites but also for surrounding tissues (77–80) (**Figure 1**).

P2 RECEPTORS EXPRESSED BY EOSINOPHILS

There is currently no systematic study on the expression of P2 receptor specific mRNAs and proteins in eosinophils. Another source of uncertainty is represented by the fact that the expression of individual P2 subtypes is not replicated in all studies. This bias can be due to the presence (or absence) of contaminating cells in different eosinophil preparations and/or to sensitivity of the techniques used. Different studies revealed that human, murine and rat eosinophils express mRNAs for different P2X and P2Y receptors including P2X1, P2X4, P2X5, P2X7, P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 (81–85). An RNASeq study confirmed expression of P2Y6 and P2X5 mRNAs (86). Proteomic studies have shed light on expression of P2 receptor proteins, showing that human blood eosinophils express P2Y2, P2Y4, P2Y13 and P2Y14 as well as P2X1, P2X2 (87). Several pharmacologic studies performed with P2 receptor agonists and antagonists confirmed functionality of the receptors on eosinophils. Interestingly, currents evoked by the P2X agonist alpha, beta-methylene ATP were lower in eosinophils derived from asthmatic subjects compared to eosinophils derived from healthy donors, although P2X1 mRNA and protein expression was comparable in both groups. However, this effect in eosinophils isolated from asthmatics was negated by pharmacological degradation of extracellular ATP using apyrase, suggesting that P2X1 receptors were partially desensitized due to ATP release by eosinophils and raising the question why eosinophils from asthmatic subjects might release the nucleotide (88).

NUCLEOTIDE MEDIATED RESPONSES IN EOSINOPHILS

Eosinophils are activated by a plethora of soluble mediators including cytokines such as IL-3, IL-5, IL-8, and GM-CSF, CC- chemokines, complement factors C3a and C5a, PAF, prostaglandin D2 (PGD2) and LPA, leukotriene B4 (LTB4) (59, 66, 89–91). Moreover, eosinophils respond to alarmins released by damaged tissue during infection or inflammation and stimulate immune responses and tissue remodeling (92). Nucleotide stimulation of human eosinophils was reported almost 30 years ago when it was shown that extracellular ATP secreted by thrombin-stimulated platelets exerted chemoattractant effects on human eosinophils (93, 94). Of note, the interaction of platelets and eosinophils contributing to tissue inflammation and remodeling was demonstrated in later studies (95–97).

In addition, eosinophils are also capable of secreting ATP which in turn autocrinally stimulates the release of different pro-inflammatory mediators by activating P2Y2 receptors (98) (**Figure 2**).

EOSINOPHILIC TISSUE INFILTRATION

Airway infiltration by eosinophils is driven by binding of the cell surface molecule α4β1 integrin (VLA-4) on eosinophils to VCAM-1. Accordingly, mice deficient for VCAM-1 fail to develop pulmonary eosinophilia (99, 100). Extracellular nucleotides (ATP, UTP) have been implicated in modulating the expression and function of adhesion molecules including VCAM-1 (101, 102). In this context, P2Y2 receptor signaling might play an important role since it has been shown to modulate both membrane-bound and soluble VCAM-1 in a mouse model of OVA-induced lung inflammation. Furthermore, P2Y2-deficiency in the same model was associated with a reduced VCAM-1 up-regulation and lung eosinophilia compared to wild type animals (84).

In addition to VCAM-1, eosinophils express the integrin family member CD11b. CD11b interacts with CD18 to form the complement receptor 3 (CR3) heterodimer, which also contributes to eosinophil migration into inflamed tissue. Endothelial cells release ATP in response to different stimuli which might modulate the expression and function of CD11b and other adhesion molecules in circulating granulocytes (103, 104). Hence, *in vitro* stimulation of human eosinophils with ATP results in a fast (within seconds) and dose-dependent up-regulation of CD11b (105). In line with this, exposure of human eosinophils to pharmacological P2X and P2Y agonists induces

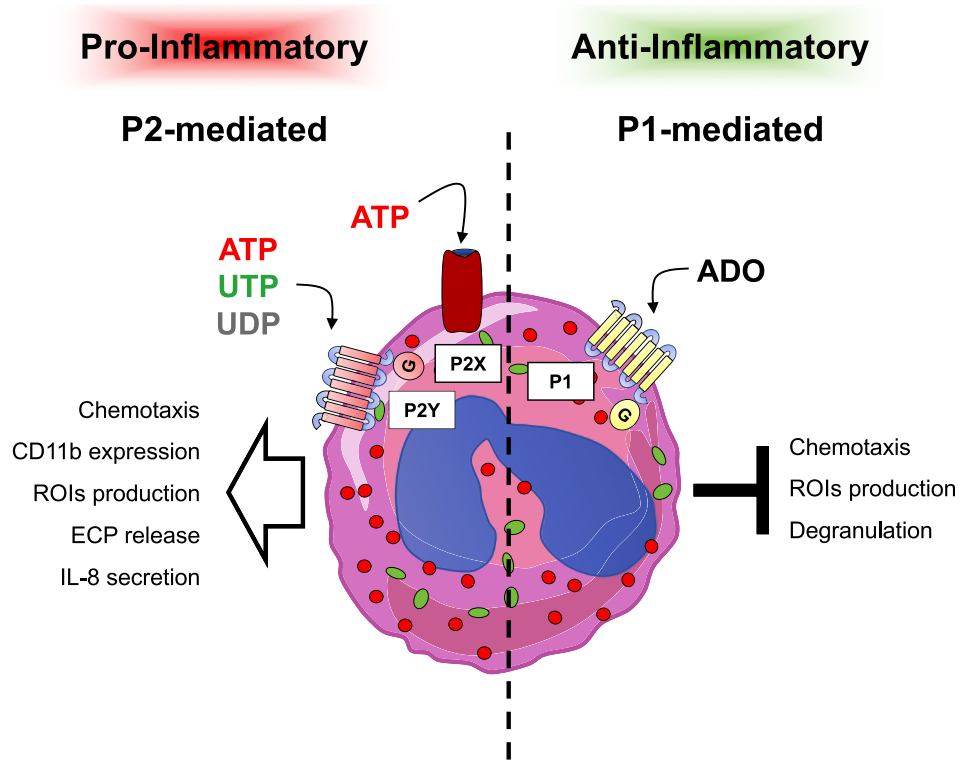

FIGURE 2 | Responses induced by extracellular nucleotides and adenosine in eosinophils. Eosinophils express P1 and P2 receptor subtypes whose stimulation has been linked to different responses. In particular, pro-inflammatory P2-mediated responses (left part) confer to eosinophils a pro-inflammatory behavior, while on the contrary, anti-inflammatory P1-mediated responses (right part) induce anti-inflammatory effects.

CD11b expression (106). The fast kinetic suggests a nucleotide-mediated plasma membrane trafficking by intracellularly stored CD11b rather than an induced transcription of the *cd11b* gene which would be delayed. The P2X1 receptor subtype seems to be crucial in this context, since the P2X1 activation using alpha,beta-methylene ATP promotes αMβ2 integrin–dependent eosinophil adhesion. This effect was higher in eosinophils from healthy individuals compared to patients suffering from asthma (88).

Apart from modulating the expression of adhesion molecules, a direct chemotactic P2Y2-dependent effect of ATP on eosinophils has been demonstrated. Of note, eosinophils derived from asthmatic patients showed an up-regulation of P2Y2 receptor expression accompanied by an increased ATP-driven migration (22, 106) (**Figure 2**).

RELEASE OF EFFECTOR MOLECULES/PRO-INFLAMMATORY MEDIATORS

Activated by different stimuli such as eotaxin and complement proteins C3a and C5a, human eosinophils generate reactive oxygen intermediates (ROIs). Extracellular nucleotides (ATP, ADP, UTP, GTP, and BzATP) have also been implicated in the production of ROIs via activating both P2X and P2Y receptors (83, 106). In accordance, blood eosinophils isolated from asthmatics showed an increased expression of P2X7 receptors compared to healthy controls. Simultaneously, these eosinophils produced higher amounts of ROIs after stimulation with the P2X7 agonist BzATP (107).

Eosinophil granules contain different enzymatic and non-enzymatic proteins promoting host defense but also the pathogenesis of chronic diseases such as asthma, atopic dermatitis, prurigo nodularis and vasculitis, where they cause tissue damage associated with inappropriate release. Eosinophil cationic protein (ECP) is a known marker of eosinophil activation/participation under pathophysiological conditions (108). Stimulation of eosinophils with ATP, UTP and UDP, but not BzATP, ADP or alpha,beta-methylene ATP induces the release of ECP in a dose-dependent and pertussis toxin sensitive manner. This suggests the involvement of P2Y receptors, potentially of the P2Y2 subtype, in purine-driven ECP release. Similar observations have been made for the eosinophil derived neurotoxin, a protein closely related to ECP with cytotoxic properties, which is released following P2Y2 receptor activation (83, 109). Human and mouse eosinophils also express the P2Y12 receptor. Accordingly, ADP stimulated secretion of eosinophil peroxidase (EPO) in a P2Y12 dependent manner in human eosinophils has been shown (85).

Interleukin-8 (IL-8) or CXCL8 is a human chemokine produced by innate immune cells including eosinophils but also by endothelial or epithelial cells. Augmented IL-8 secretion

has been observed in eosinophils from patients with asthma or atopic dermatitis (110, 111). Human eosinophils secrete IL-8 in response to stimulation with UDP, ATP, alpha,beta-meATP, and BzATP, while UTP or ADP show no effect. From the pharmacological profile of the response and use of P2 inhibitors, both P2Y and P2X receptor subfamilies could be involved in IL-8 secretion. A participation of P2Y6, P2X1 and P2X7 subtypes has been hypothesized (109). Moreover, a recent study demonstrated that release of pro-inflammatory cytokines by human eosinophils upon stimulation with the endogenous danger signal crystalline uric acid is dependent on autocrine secretion of ATP in the extracellular space and on the expression of purinergic receptors (**Figure 2**) (98).

P1 RECEPTORS EXPRESSED BY EOSINOPHILS AND THEIR RESPONSES

Adenosine P1 receptors have been shown to strongly suppress eosinophil pro-inflammatory functions. In asthma, the anti-inflammatory effects of the drug theophylline are enhanced by A3 receptors expressed on eosinophils. Accordingly, ADO administration boosts the beneficial effects of clinically relevant theophylline concentrations, while administration of the selective A3 antagonist MRS 1220 alleviates the anti-inflammatory effects of theophylline. However, A1 and A2 antagonists fail to inhibit theophylline treatment (112).

Furthermore, A3 activation in eosinophils triggers Ca^{2+} release from intracellular stores (113, 114). However, A3 activation does not appear to be a prime mechanism for free radical generation by human peripheral blood eosinophils and an inhibitory effect of A3 receptor subtypes on the degranulation of human eosinophils and O^{2-} release has been suggested (115, 116). The same adenosine receptor has been found to have a regulatory function on the migration of eosinophils to the site of inflammation. *In vitro* experiments revealed that the A3 receptor signaling inhibits the migration of human eosinophils in response to PAF, RANTES, and LTB4 (117). This inhibitory effect has been confirmed *in vivo*, where A3 activation significantly reduces PAF-induced eosinophil migration to the lungs. This suggests the use of A3 receptor agonists as a therapeutic approach for asthma and rhinitis (118). Of note, an atypical form of the A3 receptor found in human eosinophils is positively coupled to AC and promotes anti-inflammatory responses by inducing cAMP (113). In some of these human studies, expression of A3 receptor in eosinophils was determined at the mRNA level (113, 118) or by immune-labeling (116); while in most of these earlier investigations presence of A3 receptor was indirectly proven by functional or inhibition studies using selective agents or antagonists (112, 115, 117), without the expression of the receptor being proven *per se*. In recent studies, expression of ADORA2B in human eosinophils was detected by RNA-seq analysis that, however, did not detect presence of A3 receptor in the same samples (86). Further, a subsequent proteomic study by Wilkerson and colleagues, did not detect presence of P1 receptors in human eosinophils obtained from the peripheral blood. These discrepancies might result from differences in the eosinophil purification protocols or, alternatively, in the specificity of the techniques used. Further studies are needed to combine eosinophil RNA and proteomic profiling along with functional investigations, to resolve these apparent incongruities.

Under certain circumstances, selected adenosine receptors are also responsive to inosine, a purine formed by deamination and breakdown of adenosine. It has been shown that inosine contributes to lung recruitment of eosinophils in a murine model of allergic OVA-induced respiratory inflammation in an A2A- and A3-dependent manner (119) (**Figure 2**).

NUCLEOTIDE METABOLIZING ENZYMES EXPRESSED BY EOSINOPHILS

Information on expression and function of ectonucleotidases in human eosinophils are lacking.

Although expression of CD39 (ectonucleotidase-1) was demonstrated in human leukocytes from sputum and BALF, and its activity was shown to be modulated by smoking and increased in chronic obstructive pulmonary disease (COPD) (120), no clear attribution of CD39 protein to human eosinophil cells has been done so far.

In the context of asthma, some studies suggest a protective effect of global (or on regulatory T cells) CD39 expression in the modulation of eosinophil functions.

In the settings of ovalbumin-induced allergic airway inflammation, systemic CD39 inhibition by ARL67156 or through genetic deletion in regulatory T cells, worsens animal clinical conditions. Interestingly, control mice (i.e., mice with normal CD39 expression levels), present milder airway inflammation, associated with significantly lower eosinophil counts in BALF (121).

A correlation between CD39 levels in the thymus and eosinophil infiltration in the BALF has been recently observed also in experimental house dust mite (HDM)-induced allergic asthma. Results from this study suggest the beneficial effects of multiple doses of adipose tissue-derived mesenchymal stromal cells (MSCs). Notably, animals exposed to three doses of MSCs present significantly reduced inflammation in the lungs, this being associated to increased levels of CD39 in the thymus and lower eosinophil counts in the BALF (122).

CONCLUSIONS

While in healthy subjects the number of eosinophils in the peripheral blood is low, it can increase dramatically under pathophysiological conditions such as atopic dermatitis, bronchial asthma, eosinophilic esophagitis, gastritis, gastroenteritis, colitis or hematological malignancies (54, 62, 71, 80, 123). The large body of evidence supporting the critical role of eosinophils in parasitic and inflammatory diseases has prompted and intensified investigations on potential targets to modulate cellular responses of eosinophils.

Inflammation is associated with the release of nucleotides in the extracellular space, where they serve as ligands for purinergic receptors. Purinergic signaling represents an ubiquitous signal

transduction and regulatory system (7). Eosinophils express a wide range of purinergic receptors and purinergic receptor activation is associated with the recruitment of eosinophils into inflamed tissue, ROIs production, the release of effector molecules and the secretion of pro-inflammatory cytokines. Thus, the inhibition of purinergic receptors on eosinophils would be highly desirable for reducing detrimental immune responses and tissue damage related to various disorders. In accordance, blocking P2 receptors signaling using specific inhibitors or P2 receptor deficiency could be associated with decreased eosinophilic inflammation in diverse animal models. Given the wide range of specific P2 receptor inhibitors available and the successful application in clinical trials, further research in P2 inhibition as therapeutic strategy for treating eosinophilic diseases in humans is warranted.

Besides targeting eosinophil migration and activation, in inflammation another potential approach is the modulation of eosinophil-platelets interactions. In asthma patients it has been demonstrated that platelets bind to eosinophils in the blood. This event directly correlates with the occurrence of spontaneous or clinically induced (e.g., allergen challenge) asthmatic attacks (124). One randomized, placebo-controlled clinical study on the use of the anti-P2Y12 platelet inhibitor "Prasugrel" in asthmatic patients, has shown a slight, although not significant reduction, in the bronchial inflammatory burden (125).

However, other P2Y12 inhibitors (used for the treatment of thrombosis) have failed to control eosinophil recruitment in animal models of allergic inflammation (126), suggesting different levels in platelet activity in response to vascular injury, when compared to allergic responses (127). Furthermore, different studies confirmed the involvement of P2Y1 and P2Y14 receptors in platelet-dependent eosinophil recruitment in the lungs (126, 128, 129).

New developments of effective treatments for eosinophilic diseases, like asthma or allergy, are also important because eosinophils are a major source of intravascular tissue factor, a key initiator of blood coagulation (130). Disorders characterized by eosinophil accumulation have been associated with an increased risk of thrombosis. A study conducted on a cohort of patients affected by hypereosinophilia confirmed the presence of increased tissue factor expression in eosinophils from these patients compared to healthy controls (131). However, further investigation is needed to confirm whether this finding is truly associated with an increased risk of thrombosis. A comprehensive profiling of eosinophil P1 and P2 receptor expression pattern at both mRNA and protein levels would shed light on the function of these receptors in eosinophils, as well as on their biology and contribution to the regulation of pathologically relevant eosinophil responses.

Moreover, differences in the purinergic signaling of different eosinophil subpopulations could exist and be important for diseases where eosinophil participation is predominant (132, 133). Therefore, isolation of eosinophils subpopulations and analysis of their purinergic network would be requested. Another prerequisite is the characterization of the complete panel of cytokine/chemokines released by eosinophils in response to nucleotide stimulation. In future studies, it would be relevant to check the effect of ATP and other nucleotides on production of eosinophil preeminent cytokines and chemokines such as IL-5, eotaxin and RANTES. Further efforts should be done to elucidate expression and function of ectonucleotidases CD39 and CD73 in human eosinophils; this would give a more complete picture of the purinergic signaling of these cells and would help to interpret relationships between purinergic signaling in eosinophils and other cell types involved in the immune response and tissue remodeling. Eosinophils are thought to play either positive or negative roles in cancer, depending on type of tumor (59). Since nucleosides and nucleotides are present in the tumor microenvironment and heavily affect immune response against cancer, it would be worthy to check whether stimulation of the purinergic network of eosinophils modulate their responses against tumors.

AUTHOR CONTRIBUTIONS

DF, MV, ML, SR, AZ, TM, and MI conceived the review and wrote the manuscript. FC prepared the figures, contribute and revise the manuscript. EM and PS checked and revised the manuscript. All authors contributed to the article and approved the submitted version.

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OPINION
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Inflammatory Bowel Diseases: It's Time for the Adenosine System

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INTRODUCTION

The Adenosine System: Enzymes, Transporters, and Receptors

Over the years, a number of evidences have pointed out the relevant contribution of the adenosine system in the regulation of different physiological functions, highlighting a deep involvement of this nucleoside in shaping the digestive functions (1). Of note, such modulatory effects are tightly related to the levels reached by this nucleoside in the biophase of its own receptors (2). In this regard, it has been well reported that the adenosine levels vary considerably based on the health status of the tissues (3).

Under physiological conditions, low levels of adenosine are detected in the extracellular milieu, which stems mainly from the intracellular activity of S-adenosylhomocysteine hydrolase, which converts the S-adenosylhomocysteine into adenosine (1). Once synthesized, adenosine is extruded from the cells via nucleoside transporters, classified into: (a) equilibrative nucleoside transporters (ENTs), bidirectional transporters, acting on the intra- and extracellular levels reached by the nucleoside; (b) the concentrative nucleoside transporters (CNTs), promoting the intracellular influx of adenosine against its concentration gradient. Once re-uptaken intracellularly, adenosine is quickly phosphorylated into adenosine monophosphate (AMP) by adenosine kinase or deaminated into inosine via the catabolic enzyme adenosine deaminase (1). Under pathological conditions, the extracellular levels of adenosine increased markedly, mainly via the up-regulation of the ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39)-5′-nucleotidase (CD73) enzyme axis, which quickly convert the extracellular adenosine 5'-triphosphate (ATP) into adenosine (4).

The extracellular levels of adenosine are tightly controlled by adenosine deaminase which converts this nucleoside into inosine, and then to the end product uric acid via xanthine oxidase (5). In parallel, adenosine kinase also takes part to finely tune the extracellular adenosine concentration, phosphorylating it once recovered inside the cell (1).

The physiological and pathophysiological activity of adenosine are mediated by the engagement of four specific G-protein-coupled receptors named A_1, A_{2A}, A_{2B}, and A_3 (6). The A_1 and A_3 receptors, once stimulated, induce an intracellular release of calcium via interaction with G_i, G_q, and G_o proteins (7). The A_{2A} and A_{2B} receptors, related with G_s or G_{olf}, activate adenylyl cyclase (7). Of note, A_{2B} receptors can also elicit the activation of the phospholipase C via G_q protein (7).

Adenosine System in IBD Pathophysiology

IBDs are chronic relapsing disorders affecting the digestive tract, clinically classified as Crohn's disease or ulcerative colitis based on symptoms, disease location, and histopathological features (8).

A common denominator observed in IBD patients is deregulated intestinal mucosa functions as well as an exuberant activity of immune cell populations (9, 10). Indeed, in IBD patients

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the barrier function appears critically compromised, thus leading to an increased permeability to noxious intraluminal stimuli. In particular, the bacteria, overwhelming the intestinal barrier, infiltrate the *lamina propria* triggering the mucosal immune system activity and spurring the inflammatory process (11). In parallel, the IBD patients display both a T cell dysfunction as well as an antigen-presenting cell alteration (9, 10).

Clinically, it has been reported that Crohn's disease can affect any part of the digestive tract (12). In particular, this disorder is characterized by "patches," affecting some areas of the gut, leaving other sections completely unaltered (12). Histologically, Crohn's disease displays a transmural inflammation of the bowel wall (12). By contrast, ulcerative colitis is limited to the colon and the rectum, with an inflammation occurring only in the innermost layer of the lining of the intestine (12).

Immunologically, Crohn's disease is characterized by a T_{H1}/T_{H17} paradigm, leading to a marked release of IL-1β, IL-6, IL-12, IL-17, IL-21, IL-22, IL-23, IL-26, TNF, and IFN-γ (13). The ulcerative colitis patients showed a T_{H2}/T_{H9} paradigm, which determines a massive production of IL-4, IL-5, IL-9, IL-13, and IL-25 (13).

Over the years, the increasing availability of different preclinical models of IBDs, allowed a better understanding of the pathophysiological mechanisms underlying these diseases (14). At present, more than 60 animal models have been established to study IBD, distinguished in chemically induced, congenial mutant, cell-transfer, and genetic models (14). Among the chemical-induced colitis models, the dinitro- or trinitrobenzene sulfonic acid (DNBS or TNBS, respectively), oxazolone and dextran sulfate sodium (DSS)-induced colitis are widely employed (15). DNBS- or TNBS-administration elicited a T_{H1}/TH_{17} immune response, closely mimicking the Crohn's disease features (15). The DSS-induced colitis, despite being widely employed, is a spurious model, displaying a T_{H1}/T_{H2} cytokine pattern (15). By contrast, ulcerative colitis in humans is well mimicked by the oxazolone-induced colitis, which typically exhibits a T_{H2} immune response (15).

Among the genetically engineered murine models of colitis, the IL-10-knockout mice spontaneously develop a transmural pancolitis and cecal inflammation, similar to human Crohn's disease (14). In addition, the adoptive transfer models, induced by the selective transfer of immune cell types, usually $CD4^+$ T cells, in immunodeficient animals, provided relevant information about the role of T cells in shaping the mucosal immunity (14).

In parallel, the availability of a number of cell culture systems allowed to *in vitro* dissect the relevance of various cell populations in IBD onset and development (16). However, the cell culture models display several points of criticisms. Indeed, most of the cell lines employed are often immortalized neoplastic cell lines (16). In particular, the colonic cell lines Caco-2, HT29 and T84, despite displaying morphological and functional features of differentiated intestinal epithelial cells, they are characterized by neoplastic features in terms of phenotype and metabolism, thus not adequately representing the physiological or the inflammatory condition (16). In this regard, the primary human intestinal epithelial cells obtained from healthy subjects or IBD patients should be the most representative model, but unfortunately their employment is complicated by the extreme phenotype variability and by the reduced viability once in culture (16).

Over the years, several evidences highlighted a critical role of adenosine in the maintenance of intestinal homeostasis, and in orchestrating the interplay between the intestinal epithelial cells, the neuromuscular compartment and the enteric immune system (1). In particular, adenosine and its receptors demonstrated a profound reorganization in the inflammatory contexts, taking a significant part in shaping the immune responses (17). On these premises, several studies investigated the therapeutic potential of ligands acting on the adenosine system in the management of intestinal inflammation (17). However, a critical evaluation of the available pre-clinical studies about the efficacy of drugs acting on the adenosine system in managing the chronic inflammatory bowel diseases, is complicated by the heterogeneity of the *in vivo* and *in vitro* models employed, which could lead, in some cases, to conflicting results.

Role of Adenosine System in Intestinal Inflammation

Crohn's patients with active disease displayed an increased A_{2A} receptor mRNA expression in colonic mucosa, while no changes were observed in patients with ulcerative colitis (18). Conversely, others reported a decreased mRNA and protein expression of A_{2A} receptor in sigmoid colonic mucosa from active ulcerative colitis patients (19, 20). In addition, the authors observed that A_{2A} receptor expression was oppositely related with miR-16 expression (19). In particular, miR-16, targeting the 3′-UTR of A_{2A} receptor mRNA, has been found to inhibit A_{2A} receptor transcription (19). Zhang et al. (20) observed a correlation between the increase in miR-15 and a decreased expression of A_{2A} receptor mRNA in colonic tissues from ulcerative colitis patients. The authors demonstrated in HT-29 cell lines that miR-15 downregulated A_{2A} receptor mRNA expression, which, in turn, decreased the activation of pro-inflammatory NF-κB signaling (20).

Several studies showed that in the presence of bowel inflammation, A_{2A} receptors critically regulate T cell functions. Naganuma et al. (21) reported that co-transfer of $CD45RB^{low}$ or $CD25^+$ Th cells lacking A_{2A} receptors to immunodeficient mice transferred of pathogenic $CD45RB^{high}$ Th cells failed to prevent disease. Conversely, co-transfer of *wild-type* $CD45RB^{low}$ or $CD25^+$ Th cells prevented the onset of the disease, revealing a critical involvement of A_{2A} receptor in the onset of experimental colitis.

The pharmacological activation of A_{2A} receptors, via inosine administration, has exerted beneficial effects in animals with colitis induced by TNBS, indicating the A_{2A} receptor activation as an intriguing pharmacological strategy for management of gut inflammation (22). Likewise, oral administration of PSB-0777, a scarcely absorbed A_{2A} receptor agonist, alleviated bowel inflammation in oxazolone-induced colitis rats (23). However, treatment with CGS21680, a recognized selective A_{2A} receptor agonist failed in ameliorating a murine model of DSS-induced colitis (24).

The discrepancy in term of efficacy about the pharmacological A_{2A} receptor stimulation in the murine models of colitis, could be ascribable to the difference in the pathophysiological mechanisms underlying such experimental models. Indeed, it has been widely recognized that the T cells play a relevant role in the onset and development of TNBS or oxazolone colitis (25, 26), but not in DSS colitis (27). Of note, the main immunomodulatory action of A_{2A} receptors is mainly targeted to T cell population and only marginally on other immune cell populations (28). In line with this evidence, a number of data showed a lack of efficacy of CGS 21680 in stemming the phlogistic process, such as the DSS colitis, mainly driven by macrophages (29, 30).

Besides A_{2A} receptors, enteric immune and non-immune cells, with particular regard for intestinal epithelial cells, express A_{2B} receptors (31, 32). Indeed, both patients and mice with colitis displayed an increased A_{2B} receptor expression in the intestinal epithelial cells (33). In this context, A_{2B} receptors hold a key role in the maintenance of gut epithelial barrier integrity and functions, through the regulation of secretory activity, permeability and interaction with bacteria, pivotal factors implicated in IBD (33). Of note, the endothelial cells and the macrophages also showed the presence of A_{2B} receptors (34). Previous studies showed that the pharmacological block or gene deletion of A_{2B} receptor ameliorated the colitis in mice (35, 36). Conversely, Frick et al. reported that both the genetic or pharmacological ablation of A_{2B} receptors augmented the course of colitis, thus suggesting a protective role for A_{2B} receptors (37). In addition, they demonstrated that mice with A_{2B} receptor gene deletion in intestinal epithelial cell were less susceptible to the development of bowel inflammation, thus confirming a pivotal role of A_{2B} in the protection against colitis, suppression of inflammation as well as in preserving intestinal barrier integrity (38). These conflicting data regarding the role of A_{2B} receptors in bowel inflammation could result from different experimental designs, environmental variability and differences in knockout murine strains, including variation in bacterial flora composition.

Of interest, a role of A_3 adenosine receptors in the pathophysiological mechanisms of IBDs has also been described. Indeed, both patients with ulcerative colitis and in animals with experimental colitis displayed a decrease in A_3 receptor expression in colonic tissues (18, 39, 40). However, others reported an increased level of A_3 receptors in peripheral blood mononuclear cells of Crohn's patients (41). In a recent study, Ren et al. (42) showed that patients with ulcerative colitis were characterized by a decreased A_3 receptor expression along with an increase in TNF and IL-1β concentrations as well as NF-κB p65 expression in colonic mucosa. The pharmacological stimulation of A_3 receptors via 2-Cl-IB-MECA reduced the TNF and IL-1β levels and counteracted the NF-κB p65 activation in colonic tissues from UC patients, thus suggesting a role of A_3 in the pathogenesis of bowel inflammation (42). Mabley et al. (43) demonstrated that IB-MECA treatment to DSS mice, TNBS rats as well as IL-$10^{-/-}$ animals exerted beneficial effects on bowel inflammation, ameliorating the clinical symptoms and histological signs of inflammation and suppressed inflammation (43, 44). Conversely, gene deletion of A_3 receptor in mice was associated with a lower susceptibility to the development of colitis induced by DSS (40).

Such conflicting findings could be ascribed to different experimental conditions, including differences in gut microbiota composition, regarded as an important factor in the development of colitis (45). In addition, it is worth noting that the ablation of A_3 adenosine receptors determines an upregulation of other adenosine receptors, such as A_{2A}, which, in turn, exert protective effects in bowel inflammation (21).

Of interest, besides the adenosine receptors, the CD39/CD73 axis, involved in the adenosine synthesis, is emerging as a novel pharmacological target in IBD (4).

Gibson et al. observed a decreased expression of CD39 on T_{regs} from IBD patients when compared with healthy subjects (46). In addition, the authors reported that treatment with the anti-TNF infliximab determined an increase in CD39 expression on T_{regs} (46). Bai et al. observed a decreased expression of CD39 in a Th17 subpopulation with suppressor activity of patients with IBDs (47). Others reported an increase in $CD39^+CD8^+$ T cells in peripheral blood as well as in the *lamina propria* of Crohn's disease patients (48). Both $CD39^+$ Th17 and $CD39^+CD8^+$ T cells have been found to exert immunosuppressive effects through the production of adenosine (48). Confirming the immunosuppressive role of CD39 in IBDs it has been observed that a single nucleotide polymorphism determining low levels of CD39 expression was related with a higher susceptibility to the development of Crohn's disease in a case-control cohort including 1,748 IBD patients and 2,936 controls (49). Taken together, these findings suggest a protective role of CD39 in patients with IBDs.

To better understand the role of CD39 in the pathogenesis of bowel inflammation has been well characterized by means of animal models of colitis. Friedman et al. showed that $CD39^{-/-}$ mice displayed an enhanced inclination to DSS-induced colitis. Such an effect was rescued by the administration of exogenous ATPase apyrase (49). Conversely, others observed that TNBS mice with CD39 gene deletion were characterized by a lower severity of colitis as compared with wild type TNBS animals (50). In addition, they observed that the severity of oxazolone-induced colitis was comparable in CD39 KO mice as well as in wild-type animals (50). The explanation for these heterogenous results could be ascribed to the different experimental models of colitis employed. Indeed, the TNBS model shows clinicopathological features reminiscent to Crohn's disease while oxazolone-induced colitis resembles ulcerative colitis (26). However, further investigations in mice with cell-specific and temporal targeting of CD39 are needed to clarify the involvement of CD39 in bowel inflammation.

A critical role for CD73 in maintaining intestinal homeostasis has also been described (51–54). Doherty et al. displayed that patients with IBD were characterized by an increased numbers of circulating and colonic $CD73^+CD4^+$ T cells during the active phase of inflammation and such an increase was counteracted following anti-TNF treatment (55). In addition, patients with active IBD displayed an increase in CD73 on Th_{17} cells (55).

In order to better clarify the role of CD73 in the onset of bowel inflammation, several studies have been carried out in

pre-clinical models of colitis. One of these works demonstrated an increased expression of CD73 in colonic mucosa of mice treated with TNBS (51). In addition, the induction of colitis in $CD73^{-/-}$ mice was associated with worsening clinical course and inflammation. On the same line, the pharmacological blockade of CD73 with the selective inhibitor α,β-methylene ADP increased the severity of colitis in *wild type* TNBS mice (51). In addition, Bynoe et al. demonstrated that the protective effects of CD73 in bowel inflammation resulted from the induction of IFN-αA, whose administration reversed the deleterious CD73 phenotype (56). However, the activation of CD73 on T_{regs} was not dispensable for its protective effects in bowel inflammation. Moreover, the co-transfer of *wild-type* T_{regs} to $Rag^{-/-}$ mice exerted beneficial effects on bowel inflammation comparable to co-transfer of CD73 deficient T_{regs} (56). Based on these data, it is evident that the relevance of CD73 in the pathophysiology of the intestinal inflammation. In particular, by means of CD73 knockout mice, it has been demonstrated that a reduced expression of this enzyme in effector immune cells contribute to the IBD pathogenesis. Moreover, the critical role of CD73 in the maintenance of the colonic epithelium integrity has been also observed, as corroborated by the marked degree of colonic inflammation and tissue damage in CD73 knockout mice.

Growing evidence highlights an involvement of ADA in the IBD pathophysiology (5). Maor et al. showed that Crohn's patients during the active phase of the disease displayed higher circulating ADA and ADA2 levels in comparison with patients in remission as well as in healthy subjects (57). In addition, the increased circulating ADA levels in patients with ulcerative colitis were found to correlate with the severity of the disease (58). An enhanced expression of ADA was also observed in animal models of experimental colitis (59, 60). Of note, treatment with ADA inhibitor alleviated the severity of inflammation in animals with colitis (60–63). These findings suggest that ADA could represent a potential diagnostic marker as well as therapeutic targets in the treatment of IBDs. Indeed, the simplicity to evaluate the ADA expression and activity associated with a good cost effectiveness ratio represent elements in favor of using this enzyme as a useful inflammatory biomarker in IBD patients, despite this additional controlled studies are needed to further corroborate the role of ADA as an independent index of inflammation in IBDs.

As previously described, the nucleoside transporters actively participate in maintaining the adenosine levels in the extracellular space. In this regard, Wojtal et al. observed that colonic tissues obtained from patients with IBDs displayed increased mRNA levels of ENT1, ENT2, and CNT2 mRNA, thus leading to hypothesize a reduced bioavailability of endogenous adenosine (64). Interestingly, Aherne et al. reported that the administration of dipyridamole, a ENT 1 and ENT2 blocker, exerted protective effects in a murine DSS model of colitis (65). In this context, the ENT1 gene deletion did not counteract the progression of colitis, while ENT2 gene deletion was protective against intestinal inflammation, suggesting a critical involvement of ENT2 in the onset and development of bowel inflammation (65). The mechanisms underlying the anti-inflammatory effects of ENT2 inhibition or deficiency resulted from the increased levels of extracellular adenosine that exerted its protective effects through A_{2B} receptor activation (65). Unfortunately, no data are available about the beneficial effects of a pharmacological modulation of ENT2 in other murine models of colitis, not allowing a comprehensive evaluation of its efficacy in intestinal inflammation supported by other immune paradigms.

Overall, current human and pre-clinical evidence support the contention that pharmacological modulation of purinergic pathways is a suitable therapeutic approach for the treatment of bowel inflammation. In particular, A_{2A} and A_3 receptor agonists displayed beneficial effects in intestinal dysfunctions associated with inflammatory bowel disorders, including visceral pain, diarrhea, ischemia and functional disorders. However, the role of purinergic system in the modulation of digestive functions still remains poorly understood and deserves extensive future investigations.

Role of Adenosine System in Abdominal Pain

Abdominal pain is a symptom frequently associated with the presence of IBDs (66). Indeed, a number of patients in the acute phase of IBD will experience pain, typically improving upon disease activity decrease (66). Of note, a large part of IBD patients continue experiencing pain also under clinical remission (66).

Over the past years, huge efforts have been addressed to characterize the role of the endogenous mediators released during enteric dysfunctions and involved in pain perception (1). In this regard, adenosine receptors are actively involved in the rearrangement of enteric sensory pathways (1).

At present, the role of adenosine in the pathophysiology of visceral pain has been scarcely deepened and often the available evidences are conflicting (67, 68). Pre-clinical studies pointed out an inhibitory effect exerted by adenosine, via A_1 receptor activation, on pain transmission both at pre-synaptic level, counteracting the pain-associated neurotransmitter release, such as glutamate, calcitonin gene-related peptide and substance P, and at post-synaptic level, through membrane cell hyperpolarization (68, 69). Sohn et al. reported that the intrathecal administration of the A_1 receptor agonist R-PIA, but not the A_{2A} receptor agonist CGS-21680 hydrochloride, decreased the visceromotor responses (70). Currently, some authors paid greater attention to the potential anti-nociceptive effects of A_3 agonists (71, 72). For instance, Hou et al. demonstrated the analgesic effects of A_3 receptor agonists in a mouse model of visceral pain following experimental colitis (71). At present, no data are available about the putative analgesic effect of A_{2A} ligands on abdominal pain associated with experimental colitis. This is an intriguing point to address, since as previously described, the A_{2A} agonists are actively under evaluation for IBD management based on their marked immunomodulatory effects.

Role of Adenosine System in Enteric Dysmotility Associated With IBD

Over the years, increasing efforts have been addressed to unravel the link between the enteric inflammation and the neuronal alterations in the digestive tract. Inflammation-induced

changes occur in several neuronal compartments, including the sympathetic prevertebral ganglia, the dorsal root ganglia, and the enteric ganglia (73).

In this context, the evaluation about the involvement of adenosine pathways in the pathophysiology of enteric dysmotility associated with IBDs, has become an area of active investigation (1, 74, 75). Several evidences highlighted a marked reorganization of adenosine receptor expression and activity in the presence of intestinal inflammation (1, 17, 74, 75). Different murine model of chronic bowel inflammation revealed a reduced modulatory role by A_1 receptors in the small and large bowel (76, 77). The loss of A_1 receptor activity has been ascribed to a sustained exposure at marked concentrations of adenosine, leading to a receptor desensitization (14). A reduced inhibitory modulation via A_1 receptors on colonic cholinergic responses have been observed in a rat model of DNBS-colitis. However, the authors related this event to an increased degradation of endogenous adenosine, with a consequent reduction of its bioavailability, rather than to a receptor desensitization (14). Accordingly, Antonioli et al. reported a limited A_1 receptor activation arising from a site-specific production of adenosine, operated by the enzyme CD73, preferentially in the A_{2A} receptor biophase (14), previously reported as critically involved in the modulation of colonic nitrergic transmission in DNBS-treated rats (78). In the presence of intestinal inflammation, a reorganization of the receptor expression and function as well as the presence of functional interplays with metabolic pathways, have been described also for A_{2B} receptors (59). Indeed, the inhibitory control exerted by A_{2B} receptors on colonic contractile responses was impaired in the presence of experimental colitis, despite an up regulation of such receptors in the colonic neuromuscular layer from inflamed animals (59). Molecular investigations demonstrated the co-localization of adenosine deaminase with the A_{2B} receptor, suggesting a functional interplay, where adenosine deaminase, catabolizing the endogenous adenosine, reduced A_{2B} receptor activation (59). In accordance, adenosine deaminase has also been shown to play a modulatory role in the activity of the A_3 receptor in the inflamed colon (79).

Analogously to what was reported for A_{2B} receptors, the presence of colonic inflammation was characterized by the loss of the A_3 receptor inhibitory activity, an up-regulation of functioning A_3 receptors occurred (79). This altered A_3 receptor expression occurred concomitantly with an increase in adenosine deaminase expression in the colonic neuromuscular compartment of rats with colitis, thus decreasing the bioavailability of endogenous adenosine in the A_3 receptor microenvironment (79). Based on these evidences, the pharmacological blockade of adenosine deaminase may represent an intriguing strategy to limit the inflammatory process and contextually counteract the enteric motor alterations typically observed in IBD patients.

CONCLUDING REMARKS

The etiopathogenesis of IBD is still poorly understood, despite a number of recent evidences revealing that enhanced knowledge about the immunological mechanisms underlying IBD onset and progression represent an interesting target to design and synthesize innovative therapeutic strategies (80). The current available pharmacological options are effective, but unfortunately some of these drugs displayed marked adverse events, such as infections or an enhanced risk of neoplastic diseases or they lose their effectiveness over time. Indeed, about one third of the patients show a slight response to these therapies (80).

A number of pre-clinical studies revealed the involvement of the adenosine system in the modulation of immune, functional and sensory systems of the gastrointestinal tract (81). In this regard, an increasing interest has been focused toward the A_{2A} and A_3 receptor agonists as interesting targets to generate novel pharmacological entities useful to manage the digestive dysfunctions. Indeed, the use of selective A_{2A} or A_3 receptor agonists showed beneficial effects in counteracting the inflammatory burst in murine models of colitis, acting on both the innate and acquired component of the immune system (82–85). In parallel, the stimulation of such receptor subtype revealed to exert a significant role in the regulation of colonic neuromuscular activity in the presence of bowel inflammation (77, 79). In particular, the engagement of A_{2A} or A_3 receptors by selective agonists appear to be an interesting method of management for IBD patients displaying an increased gut motility and diarrhea (77, 79). A number of encouraging data are emerging about the modulatory role of adenosine receptors on visceral sensitivity (71, 72). The A_3 receptor agonists highlighted a pain-relieving mediated through N-type Ca^{2+} channel block and action potential inhibition, suggesting the A_3 receptor agonists as an innovative approach to manage the visceral pain (59).

These data spurred the interest of the scientific community toward the development of novel ligands acting selectively on adenosinergic receptors/enzymes. These novel pharmacological tools will allow to better deepen the pathophysiological meaning as well as the putative therapeutic relevance of the adenosine pathway, paving the way to the development of novel therapeutic options useful for the treatment of IBDs.

AUTHOR CONTRIBUTIONS

LA, MF, CP, LB, ZN, and CB participate to bibliographic research, to write and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Decreased Frequency of Intestinal CD39$^+$ γδ$^+$ T Cells With Tissue-Resident Memory Phenotype in Inflammatory Bowel Disease

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The ectoenzymes CD39 and CD73 play a major role in controlling tissue inflammation by regulating the balance between adenosine triphosphate (ATP) and adenosine. Still, little is known about the role of these two enzymes and ATP and its metabolites in the pathophysiology of inflammatory bowel disease (IBD). We isolated mononuclear cells from peripheral blood and lamina propria of the large intestine of patients diagnosed with IBD and of healthy volunteers. We then comprehensively analyzed the CD39 and CD73 expression patterns together with markers of activation (HLA-DR, CD38), differentiation (CCR7, CD45RA) and tissue-residency (CD69, CD103, CD49a) on CD4$^+$, CD8$^+$, γδ$^+$ T cells and mucosa-associated invariant T cells using flow cytometry. CD39 expression levels of γδ$^+$ and CD8$^+$ T cells in lamina propria lymphocytes (LPL) were much higher compared to peripheral blood mononuclear cells. Moreover, the frequency of CD39$^+$ CD4$^+$ and CD8$^+$, but not γδ$^+$ LPL positively correlated with T-cell activation. The frequency of CD39$^+$ cells among tissue-resident memory LPL (Trm) was higher compared to non-Trm for all subsets, confirming that CD39 is a marker for the tissue-resident memory phenotype. γδ$^+$ Trm also showed a distinct cytokine profile upon stimulation – the frequency of IFN-γ$^+$ and IL-17A$^+$ cells was significantly lower in γδ$^+$ Trm compared to non-Trm. Interestingly, we observed a decreased frequency of CD39$^+$ γδ$^+$ T cells in IBD patients compared to healthy controls (p = 0.0049). Prospective studies need to elucidate the exact role of this novel CD39$^+$ γδ$^+$ T-cell population with tissue-resident memory phenotype and its possible contribution to the pathogenesis of IBD and other inflammatory disorders.

Keywords: CD39, CD73, ATP, adenosine, γδ$^+$ T cells, gut, IBD, tissue-residency

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INTRODUCTION

Inflammatory bowel disease (IBD) is the umbrella term for Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC). These diseases share aetiological and pathophysiological features and are characterized by a combination of genetic and environmental factors causing immune dysregulation (1, 2), and altered composition of the gut microbiota (3, 4). As a result, chronic inflammation of the gastrointestinal tract occurs along with a loss of epithelial barrier

function (5). Clinically, these diseases are characterized by symptoms like diarrhoea, abdominal pain, fatigue, weight loss, and extraintestinal manifestations (6, 7).

Lymphocytes of the large intestine are exposed to high levels of extracellular adenosine triphosphate (ATP) secreted by i.a. commensal bacteria (8, 9). The balance between extracellular ATP and its metabolite adenosine has been identified as a major factor controlling inflammation in the intestinal milieu (10, 11). On the surface of T cells, ATP can bind to purinergic receptors (e.g., P2X7) leading to an increased Ca^{2+} influx and an enhanced cellular activation (12), whereas adenosine generally dampens the T-cell effector functions by binding to P1 receptors such as the A2A-receptor (11, 13). The degradation of extracellular ATP to adenosine is controlled by the ectoenzymes CD39 and CD73 (14–16). In mouse models of dextrane sulfate sodium (DSS)-induced colitis, genetic deletion of either CD39 (17) or CD73 (18) caused exacerbation of disease. In humans, single nucleotide polymorphisms of the human ENTPD1 gene that lead to a decreased expression of CD39 are associated with increased susceptibility to Crohn's disease (17). In 2018, Raczkowski et al. demonstrated that $CD39^+$ and $CD73^+$ cells in human mucosal tissue protect the epithelium from the proinflammatory effects of commensal bacteria-derived ATP in the intestinal lumen (13). Altogether, these findings strongly suggest that CD39 contributes to the regulation of the inflammatory microenvironment, and that changes in CD39 expression or function might promote the onset and perpetuation of IBD. Surprisingly, there are only few human studies that comprehensively assessed the CD39 and CD73 expression patterns of different T-cell subsets in peripheral blood and mucosal tissue of healthy individuals versus IBD patients (19, 20).

Tissue-resident memory cells (Trm) are another T-cell population that has only recently been described, which contributes to the (dys)regulation of the immunological response. These cells are particularly adapted to the intestinal niche (21–23). Specific surface markers for Trm (e.g., CD69 and CD103), as well as distinct transcriptional profiles (24) have been described for these cells. For example, it has been shown that $CD69^+$ Trm in the lung are able to initiate potent immune responses via production of IFN-γ and IL-2 while simultaneously displaying a low turnover, thereby preventing excessive inflammation (24). Thus, Trm are of particular interest in the pathogenesis of IBD and display potential new targets for therapeutic approaches (24, 25). Consequently, we wanted to re-evaluate these recent findings in the context of IBD and the contribution of ATP-converting enzymes. In particular, we investigated CD39 and CD73 expression and function of Trm to distinguish them from their recirculating counterparts.

In summary, using several comprehensive 16-color flow cytometry panels, we were able to characterize the peripheral and gut-resident immune cell compositions in healthy individuals and IBD patients. We assessed the expression of CD39 and CD73 together with markers of activation (HLA-DR/CD38), differentiation (CCR7, CD45RA), and tissue-residency (CD69, CD103, CD49a) on $CD4^+$ and $CD8^+$ T cells and non-conventional subsets like $\gamma\delta^+$ T cells and mucosa-associated invariant T cells (MAIT) in peripheral blood mononuclear cells (PBMC) and mucosal tissue. Our data hint towards a potential role of $CD39^+$ $\gamma\delta^+$ T cells with tissue-resident memory phenotype in IBD pathogenesis warranting future functional and longitudinal studies focusing on the consequences of their depletion in the mucosa of patients with IBD.

MATERIALS AND METHODS

Study Design

For this study, individuals undergoing colonoscopies were recruited at the University Medical Center Hamburg-Eppendorf. Samples from healthy subjects (n = 27) and patients diagnosed with IBD (n = 24) were obtained during regular check-up examinations or when patients were referred to the endoscopy unit for further diagnostic exploration. Four to five double biopsies from the colon mucosa were obtained with single-use biopsy forceps and directly processed afterwards. Additionally, we analyzed cryopreserved PBMC from healthy donors (n = 9), UC and CD patients (n = 10). All individuals gave written, informed consent and this study was approved by the local Institutional Review Board of the Ärztekammer Hamburg (PV5798, PV4444, PV4870) and conducted in accordance with the declaration of Helsinki. Additional information such as clinical symptoms and treatment, co-existing diseases, or the histological analysis of biopsies were extracted from the clinical data bank. Based on the data available, we evaluated the disease status for each patient (26). For an overview of the characteristics of patients who donated gut samples, see **Table 1A**, for more detailed information about the IBD patients, see **Supplementary Tables S1, S2**. An overview of the patient characteristics of the analyzed PBMC samples can be found in **Table 1B**. For a more detailed description, see **Supplementary Table S3**.

Sample Acquisition and Processing

Collected in sterile PBS, the samples were processed as previously described (27, 28). In brief, after incubation in Hank's Balanced Salt Solution (HBSS) containing DTT and EDTA for a short digestion period, they were stored in 6-well, low-binding plates overnight in RPMI supplemented with 10 % FCS, antibiotics and antifungals (1 mg/mL Piperacillin/Tazobactam and 1.25 μg/mL Amphotericin B). The next day, the remaining tissue was disrupted by pipetting and filtered through a 100 μm nylon mesh. After that, the isolated mononuclear cells from the lamina propria were stained and measured immediately. In some cases, the isolated lamina propria lymphocytes (LPL) were cryopreserved and stained later due to the organizational set-up. Frozen PBMC were thawed and stained directly.

Immune Phenotypic Analysis of Surface and Intracellular Markers of Different Lymphocyte Subsets

Cells were stained with Zombie NIR Fixable Viability stain (BioLegend) and fluorochrome-conjugated antibodies (**Supplementary Table S4**).

TABLE 1 | Basic and clinical patient characteristics.

(A) LPL donors

Characteristics	HD	CD	UC	IC
n	30	13	15	1
Female/male	16/14	7/6	9/6	0/1
Median age at sampling (RANGE)	61.3 (34–90)	38.38 (23–58)	45.67 (21–66)	31
Disease specific medication				
Mesalazine	-	5	14	1
Azathioprine	-	2	2	1
Corticosteroids	-	1	2	-
Mercaptopurine	-	1	-	
Anti-TNF-α	-	3	1	-
Anti-IL-12 + Anti-IL-23	-	2	-	-
Anti-α4β7	-	1	1	-

(B) PBMC donors

Characteristics	HD	CD	UC
n	9	5	5
Female/male	5/4	2/3	1/4
Median age at sampling (RANGE)	25,33 (23-29)	48 (28-69)	38,4 (27-54)
Disease specific medication			
Mesalazine	-	-	2
Azathioprine	-	1	1
Corticosteroids	-	2	3
Anti-α4β7	-	3	1
Anti-TNF-α	-	1	-

HD, healthy donors; CD, Crohn's disease; UC, ulcerative colitis; IC, indeterminate colitis; LPL, lamina propria lymphocytes; PBMC, peripheral blood mononuclear cells.

Certain samples were stained intracellularly as well using the FOXP3 Fix/Perm buffer set (eBiosciences, San Diego, CA, United States) (29) according to the manufacturer's protocol. The samples were stained with the following fluorochrome-labeled antibodies: anti-FOXP3 (AF647, clone: PCH101, eBiosciences, San Diego, CA, United States), anti-IL17A (BV605, clone: BL168), anti-IFN-γ (PE/Dazzle 594, clone: Mab11), anti-IL-10 (BV421, clone: JES3-907), anti-CD4 (PerCP-Cy5.5, clone: SK3) (all Biolegend, London, United Kingdom). For compensation of the panels, single-stained CompBeads (Anti-Mouse Ig,κ/Negative Control Compensation Particles Set, BD Biosciences) were used. As a surrogate for the dye used for the live/dead staining, we applied the APC-Cy7 conjugated anti-CD14 antibody (Biolegend, London, United Kingdom). All samples were analyzed on a BD LSR Fortessa flow cytometer with FACS Diva version 8 (BD Biosciences) on a PC.

In vitro Stimulation

Before intracellular cytokine stainings (ICS), LPL or PBMC were stimulated with 50 ng/mL PMA and 500 ng/mL Ionomycin (Sigma-Aldrich, Seelze, Germany) and incubated at 37°C and 5% CO_2 for 5 h. For Panel B, which did not include the measurement of IL-10, we resuspended the cells in RPMI and added Brefeldin A (1 mg/mL, Sigma−Aldrich, Seelze, Germany) after 1 h. For detection of IL-10 (Panel C), we resuspended the cells in X-Vivo Medium (Lonza Walkersville Inc., United States) and after 1 h, we added Brefeldin A and Monensin (2 mM, BioLegend, London, United Kingdom). After 5 h, the cells were washed with 2 mL PBS and stained for flow cytometry. For a detailed portrayal over used LPL samples and conducted experiments, see **Supplementary Table S5**.

Data Analysis and Statistics

Cytometric data were analyzed using FlowJo v10.6.2 for Windows (FlowJo, BD, Franklin Lakes, NJ, United States). For statistical analysis, GraphPad Prism version 7.01 for Windows (GraphPad Software, Inc., La Jolla, CA, United States) was used. For multiple comparisons we computed two-way ANOVAs, whereas for single comparisons we used Mann–Whitney U tests. For matched analysis, we performed Wilcoxon matched-pairs signed rank tests. Before correlation analysis, we tested the expression of the markers analyzed for Gaussian distribution. If d'Agostino and Pearson normality test were passed, we applied Pearson's correlation and coefficient for bivariate correlation analysis. If not, Spearman correlation was implemented. In the text, we describe frequencies as means unless stated otherwise. The data on the graphs are expressed as means +/- standard deviation. A p-value equal or less than 0.05 was considered significant. p-Values are displayed as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Not significant: ns; $p > 0.05$. For the t-distributed Stochastic Neighbor Embedding (t-SNE) analysis, we used the t-SNE plugin in Flowjo version 10.6.2. Downsampling to 15,000 events was performed on seven healthy donors followed by followed by concatenation into one file for t-SNE analysis (30).

RESULTS

LPL and PBMC Differ in Their Relative T-Cell Subset Composition as Well as in Their Expression Patterns of CD39 and CD73

In a first step, we compared peripheral blood with intestinal biopsies from healthy individuals undergoing check-up colonoscopies with respect to the composition of T-cell subsets and their expression of CD39 and CD73. Flow cytometry panels were designed to differentiate between $CD4^+$, $CD8^+$, MAIT, and $\gamma\delta^+$ T cells which were further separated into $V\delta2^-$ and $V\delta2^+$ subsets for some analysis. The gating strategy is shown in **Supplementary Figure S1**. In line with other reports (31, 32), $CD4^+$ T cells were the most frequent T-cell population in both peripheral blood and the lamina propria of the gut epithelium. However, while $CD4^+$ T cells were more frequent in PBMC compared to LPL, the proportion of $CD8^+$ and $\gamma\delta^+$ T cells was higher in LPL than in PBMC (**Figure 1A**). Moreover, we observed an accumulation of $V\delta2^-$ $\gamma\delta^+$ T cells in the gut mucosa compared to peripheral blood of healthy individuals (LPL 7.05% vs PBMC 0.72%, $p = 0.0005$) (**Figure 1B**). Analysis of CD39

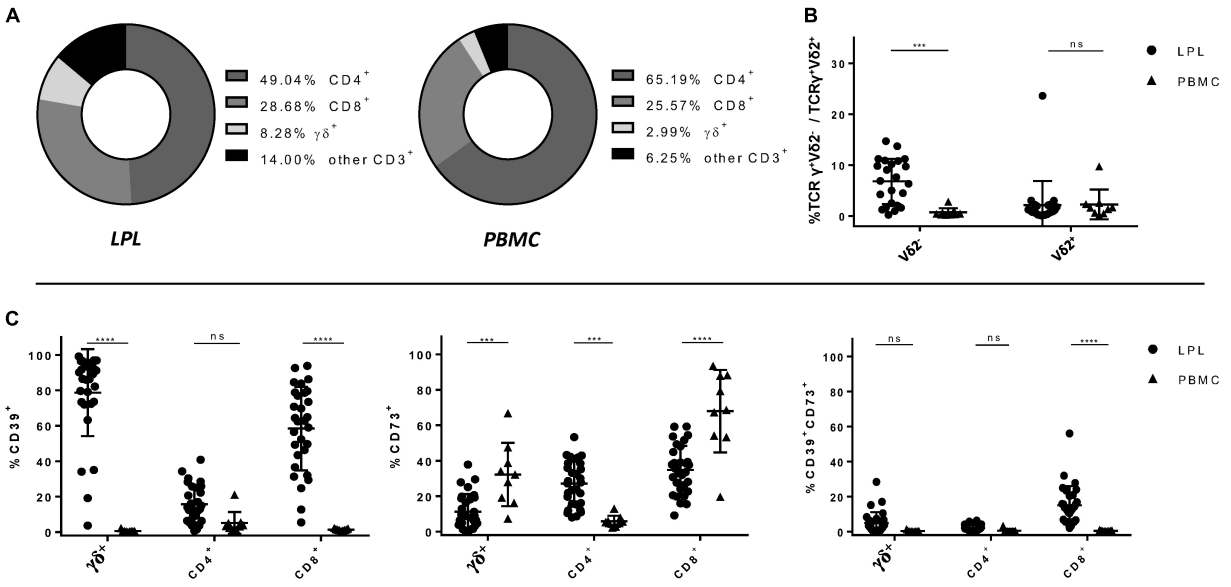

FIGURE 1 | Relative composition of T-cell subsets and frequency of $CD39^+$, $CD73^+$ and $CD39^+$ $CD73^+$ T-cell subsets in LPL versus PBMC. **(A)** Frequencies of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells isolated from lamina propria (left) and from peripheral blood (right). **(B)** Differences of the frequencies of $V\delta2^-$ and $V\delta2^+$ T cells between PBMC (round shapes) and LPL (triangular shapes). **(C)** Differences of the frequencies of $CD39^+$, $CD73^+$ and $CD39^+$ $CD73^+$ $CD4^+$, $CD8^+$, and $\gamma\delta^+$ T cells between LPL and PBMC. Data from healthy donor's LPL (n = 30/23) and PBMC (n = 9), presented as means +/- standard deviation. ns ≥ 0.05, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, as calculated by two-way ANOVA. LPL, lamina propria lymphocytes; PBMC, peripheral blood mononuclear cells.

and CD73 expression showed that the frequency of $CD39^+$ cells was significantly higher in gut-resident $\gamma\delta^+$ and $CD8^+$ T cells compared to the peripheral blood ($\gamma\delta^+$: LPL 81.23% vs PBMC 0.577%, $p < 0.0001$; $CD8^+$: LPL 59,37% vs PBMC 1.322%, $p < 0.0001$), while there was no significant difference between peripheral and gut-resident $CD4^+$ T cells (**Figure 1C**). By contrast, the frequency of $CD73^+$ cells was significantly lower on $CD8^+$ and $\gamma\delta^+$ LPL, but was increased on $CD4^+$ LPL (**Figure 1C**). The frequency of $CD39^+CD73^+$ cells was increased among gut-derived $CD8^+$ T cells in comparison to peripheral $CD8^+$ T cells. Altogether, in healthy individuals the frequency of $CD39^+$ and $CD73^+$ T cells considerably differed between PBMC and LPL for the respective subsets.

LPL of IBD Patients Show Reduced Frequencies of $CD39^+$ $\gamma\delta^+$ T Cells

Next, we compared mucosa-derived T cells of IBD patients to those of healthy individuals. We observed no significant differences in the overall frequencies of $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells, and MAIT (**Supplementary Figure S2A**). However, we observed a decreased frequency of $CD39^+$ cells among $CD8^+$ and $\gamma\delta^+$ T cells in individuals diagnosed with IBD compared to healthy donors ($\gamma\delta^+$: HD 78.36% vs IBD 57.65%, $p = 0.0049$; $CD8^+$: HD 59.37% vs IBD 47.45%, $p = 0.0325$) (**Figure 2A**). This reduction of $CD39^+$ $CD8^+$ and $\gamma\delta^+$ T cells did not seem to normalize after treatment as there was no significant difference between LPL from patients in remission and patients with intermediate or severe disease activity (**Figure 2B**). Gut-derived $CD4^+$ T cells did not show significant differences in the CD39 expression level between healthy controls and patients, but we observed a trend towards a higher frequency of $CD39^+$ $CD4^+$ T cells in patients with intermediate or severe disease activity compared to those patients who were in full remission (**Figure 2B**). In the MAIT population, there was no difference regarding the frequency of $CD39^+$ cells present (**Supplementary Figure S2B**). Furthermore, we did not observe any differences in the frequencies of $CD73^+$ and $CD39^+$ $CD73^+$ cells among the $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells, and MAIT subsets between the healthy donors and the patient group (**Supplementary Figure S3**).

CD39 Expression of Gut-Derived $CD4^+$ and $CD8^+$, but Not $\gamma\delta^+$ T Cells, Was Associated With Higher Expression of Activation Markers

Since we only observed differences in CD39 expression between healthy donors and IBD patients we put particular focus on the thorough analysis of $CD39^+$ T cells. To assess the activation status of $CD39^+$ T cells, we also analyzed the expression of traditional markers associated with T-cell activation and exhaustion (co-expression of HLA-DR and CD38, PD-1). We did not find any significant differences between $CD39^+$ T cells from healthy individuals and IBD patients, either for the frequency of $PD\text{-}1^+$ or HLA-DR/CD38 double-positive T cells (**Figure 3A**). In contrast, studying $CD39^+$ versus $CD39^-$ T cells in healthy individuals revealed significant differences: regarding gut-resident $\gamma\delta^+$ and $CD8^+$ T cells, the frequency of $PD\text{-}1^+$ cells was significantly lower among $CD39^+$ compared to $CD39^-$ cells ($\gamma\delta^+$: 2.41% vs 23.25%, $p = 0.002$; $CD8^+$: 18.01% vs 32.96%, $p = 0.0371$) (**Figure 3B**). In contrast, the

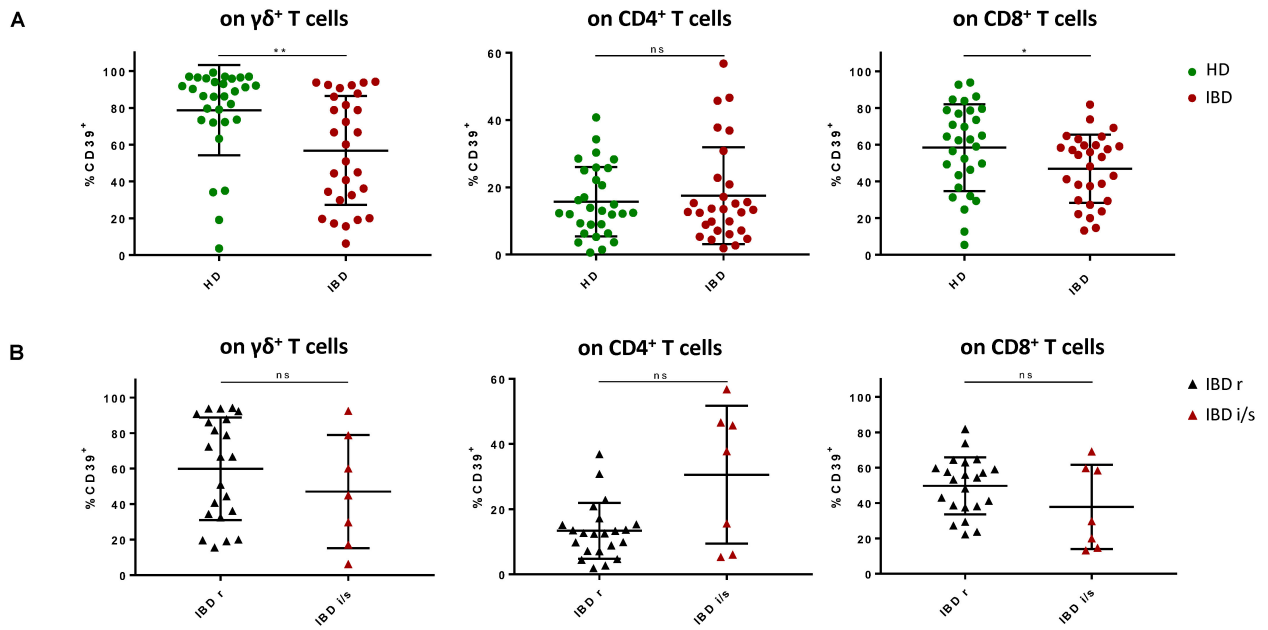

FIGURE 2 | Frequency of CD39$^+$ LPL is decreased among γδ$^+$ and CD8$^+$ T cells of patients with IBD compared to healthy donors. **(A)** Frequencies of CD39$^+$ γδ$^+$, CD8$^+$, and CD4$^+$ T cells from healthy donors (green, round shapes) and patients with IBD (red, round shapes). **(B)** Frequencies of CD39$^+$ γδ$^+$, CD8$^+$, and CD4$^+$ T cells from IBD patients in remission (black, triangular shapes) and patients with intermediate or severe disease activity (red, triangular shapes). Data from LPL of healthy donors (n = 30) and IBD patients (n = 29), presented as means +/- standard deviation. ns ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as calculated by Mann–Whitney U test. LPL, lamina propria lymphocytes; HD, healthy donor; IBD, patient with inflammatory bowel disease; IBD r, IBD patient in remission; IBD i/s, IBD patient with intermediate or severe disease activity.

frequency of PD-1$^+$ cells was significantly higher on CD39$^+$ compared to CD39$^-$ CD4$^+$ T cells (59.33% vs 43.9%, p = 0.0488) (**Figure 3B**). However, further analysis did not reveal any correlation between the expression of CD39 and PD-1 for any T-cell subset (data not shown).

With respect to T-cell activation, we observed a significantly higher frequency of HLA-DR/CD38 co-expressing cells among CD39$^+$ compared to CD39$^-$ CD4$^+$ T cells, and also a trend towards a higher frequency in the CD39$^+$ CD8$^+$ T-cell subset (CD4$^+$: 5.839% vs 1.475%, p < 0.0001; CD8$^+$: 12.19% vs 6.323%, p = 0.0797) (**Figure 3C**). Next, we performed correlation analyses to determine whether CD39 expression was associated with enhanced T-cell activation. Indeed, the frequency of HLA-DR/CD38 double-positive T cells correlated with the frequency of CD39$^+$ cells of the CD4$^+$ and CD8$^+$ T-cell compartment. Interestingly, the γδ$^+$ T cells did not show elevated levels of activation markers among CD39$^+$ γδ$^+$ T cells compared to CD39$^-$ γδ$^+$ T cells (3.611% vs 4.987%, p = 0.1729) (**Figure 3C**). There were no significant differences detectable for the frequency of HLA-DR$^+$/CD38$^+$ and PD-1$^+$ cells when we compared CD39$^+$ and CD39$^-$ T-cell subsets from healthy individuals with those from IBD patients (**Supplementary Figure S4A**). In sum, gut-derived CD39$^+$ γδ$^+$ T cells in healthy donors were characterized by low expression of HLA-DR, CD38 and PD-1. While preserving this phenotype, the frequency of CD39$^+$ γδ$^+$ T cells in IBD patients was significantly decreased compared to healthy donors.

CD39 Expression Is Associated With Different Effector Cytokine Profiles of Peripheral Versus Intestinal γδ$^+$ and CD4$^+$ T Cells

Next, we wanted to gain further insight into the functionality of CD39$^+$ LPL and PBMC. Thus, we stimulated blood- and gut-derived lymphocytes and performed ICS to assess the frequencies of IL-17A$^+$, IFN-γ$^+$, and IL-10$^+$ cells (exemplary plots: **Supplementary Figure S5**). After 5 h of stimulation with PMA/Ionomycin, peripheral γδ$^+$ T cells from IBD patients displayed significantly higher frequencies of IL-17A$^+$ cells compared to peripheral γδ$^+$ T cells from healthy donors (IBD 1.687% vs HD 0.666%, p = 0.0326) (**Figure 4A**), contrary to peripheral CD4$^+$ or CD8$^+$ T cells which displayed no differences in IL-17A expression (**Figure 4A**).

Surprisingly, the small population of peripheral CD39$^+$ γδ$^+$ T cells consisted of potent IL-17A producers with significantly higher frequencies of IL-17A$^+$ cells than in the CD39$^-$ γδ$^+$ T-cell population (healthy: 11.16% vs 0.519%, p = 0.0078). Also, CD39$^+$ CD4$^+$ T cells showed higher frequencies of IL-17A-producing cells compared to CD39$^-$ CD4$^+$ T cells (**Figure 4A**). We therefore wondered whether the gut-derived γδ$^+$ and CD4$^+$ T cells had similar characteristics. In LPL, we did not see differences in IL-17A production between healthy individuals and IBD patients for any of the subsets analyzed. As observed in PBMC, CD39$^+$ CD4$^+$ LPL displayed a higher frequency of IL-17A$^+$ cells than CD39$^-$ CD4$^+$ LPL. In contrast to PBMC, the comparison

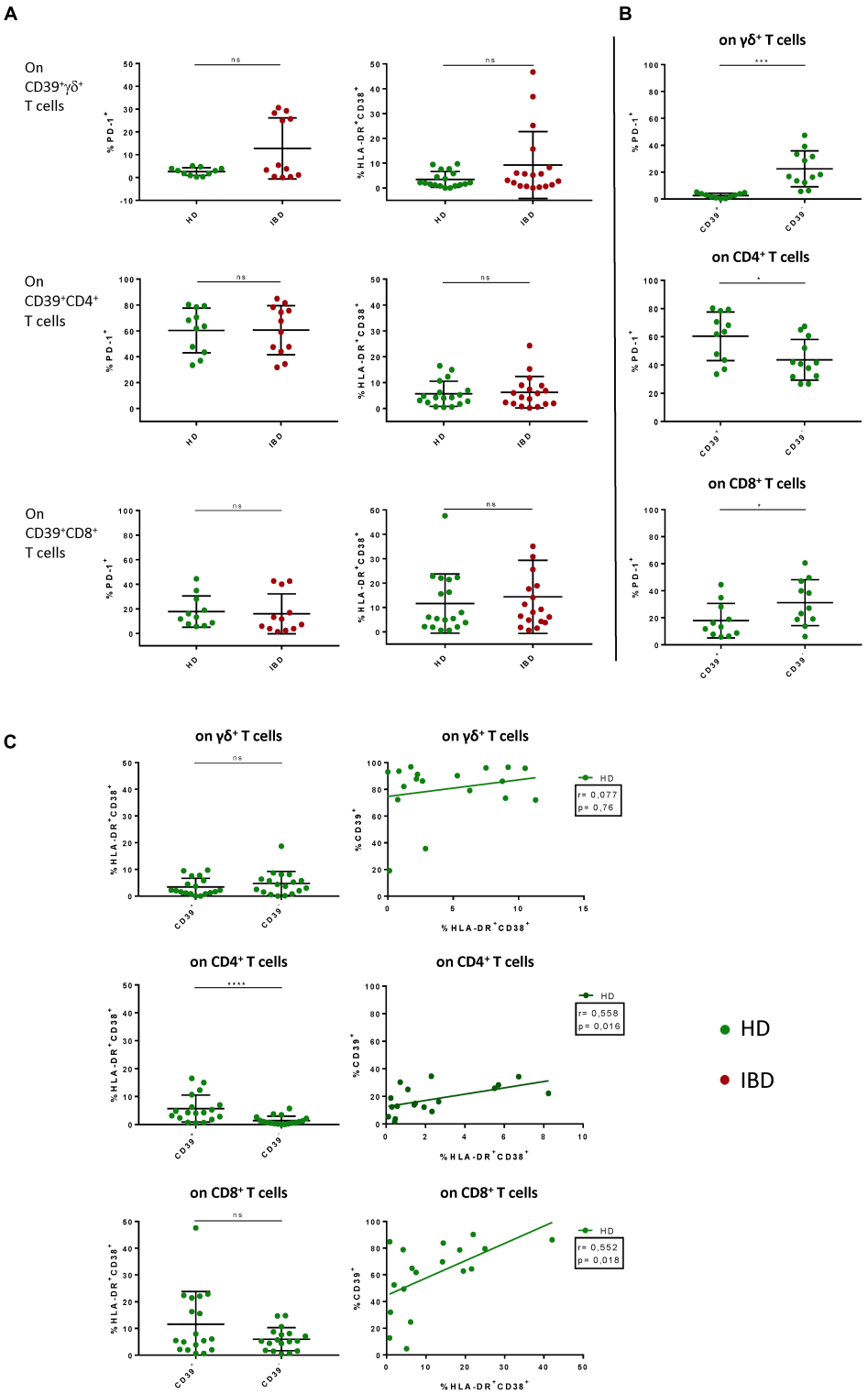

FIGURE 3 | Comparative analysis of PD-1, HLA-DR and CD38 on CD39$^+$ γδ$^+$, CD4$^+$, and CD8$^+$ T cells in LPL of HD versus IBD and CD39$^+$ versus CD39$^-$ LPL in HD. **(A)** Comparison of the frequencies of PD-1$^+$ and HLA-DR$^+$ CD38$^+$ CD39$^+$ T cells between healthy donors (green shapes) and patients with IBD (red shapes). **(B)** Comparison of the frequencies of PD-1$^+$ and HLA-DR$^+$CD38$^+$ cells between CD39$^+$ and CD39$^-$ γδ$^+$ T cells of healthy controls. **(C)** Correlation between frequencies of CD39$^+$ and HLA-DR$^+$CD38$^+$ T cells. Spearman correlation analysis was applied. Data from LPL of healthy donors (n = 11/18) and IBD patients (n = 12/18), presented as means +/- standard deviation. ns ≥ 0,05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as calculated by Mann–Whitney U test **(A)**, Wilcoxon matched-pairs signed rank test **(B)** + **(C)**, and Spearman correlation **(C)**. HD, healthy donor; IBD, patient with inflammatory bowel disease; LPL, lamina propria lymphocytes.

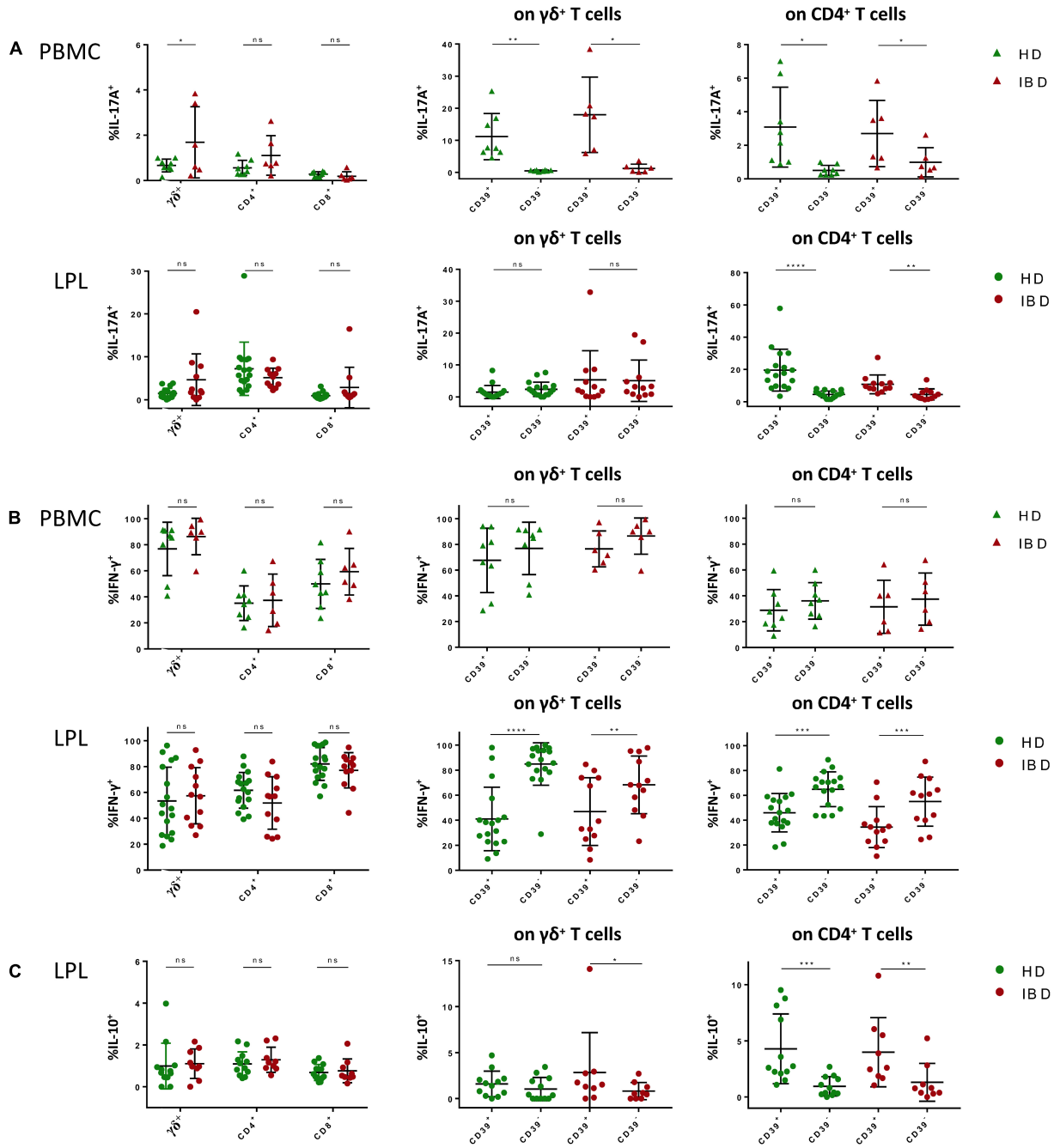

FIGURE 4 | ICS after short term stimulation of $\gamma\delta^+$, $CD4^+$, and $CD8^+$ T cells in PBMC and LPL with PMA/Ionomycin. **(A)** (Left) Frequencies of IL-$17A^+$ $\gamma\delta^+$, $CD4^+$, and $CD8^+$ T cells from healthy donors (green) and patients with IBD (red); (center) Comparison of the frequencies of IL-$17A^+$ cells between $CD39^+$ and $CD39^-$ $\gamma\delta^+$ T cells; (right) Comparison of the frequencies of IL-$17A^+$ cells between $CD39^+$ and $CD39^-$ $CD4^+$ T cells; data from PBMC in upper row (triangular shapes), data from LPL in bottom row (round shapes). **(B)** (Left) Frequencies of IFN-γ^+ $\gamma\delta^+$, $CD4^+$ and $CD8^+$ T cells from healthy donors (green) and patients with IBD (red); (center) Comparison of the frequencies of IFN-γ^+ cells between $CD39^+$ and $CD39^-$ $\gamma\delta^+$ T cells; (right) Comparison of the frequencies of IFN-γ^+ between $CD39^+$ and $CD39^-$ $CD4^+$ T cells; data from PBMC in upper row (triangular shapes), data from LPL in bottom row (round shapes). **(C)** (Left) Frequencies of IL-10^+ $\gamma\delta^+$, $CD4^+$ and $CD8^+$ T cells from healthy donors (green) and patients with IBD (red); (center) Comparison of the frequencies of IL-10^+ cells between $CD39^+$ and $CD39^-$ $\gamma\delta^+$ T cells; (right) Comparison of the frequencies of IL-10^+ cells between $CD39^+$ and $CD39^-$ $CD4^+$ T cells; data from LPL (round shapes). Data from healthy donor's LPL (n = 17/12) and PBMC (n = 8) and IBD patients' LPL (n = 11/9) and PBMC (n = 6), presented as means +/- standard deviation. ns $\geq$ 0.05, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, as calculated by two-way ANOVA and Wilcoxon matched-pairs signed rank test (comparison of $CD39^+$ and $CD39^-$ cells). HD, healthy donor; IBD, patient with inflammatory bowel disease; LPL, lamina propria lymphocytes; PBMC, peripheral blood mononuclear cells; ICS, intracellular cytokine staining.

of CD39$^+$ and CD39$^-$ gut-derived γδ$^+$ T cells did not show significant differences in the frequencies of IL-17A-producing cells.

We observed no significant difference of the frequency of IFN-γ-producing cells between peripheral CD4$^+$, CD8$^+$, or γδ$^+$ T cells of healthy individuals and the respective subsets from patients suffering from IBD (**Figure 4B**). Moreover, we did not find any association between CD39 expression and IFN-γ production in the aforementioned peripheral T-cell subsets. In LPL, there were also no differences detectable in terms of IFN-γ production between the patients and the control group for any of the subsets. However, comparing CD39$^+$ with their CD39$^-$ counterparts, CD39$^+$ γδ$^+$ T cells as well as CD39$^+$ CD4$^+$ T cells showed a significantly lower frequency of IFN-γ$^+$ cells (healthy: CD4$^+$: 47.12% vs 64.57%, p = 0.0004; γδ$^+$: 43.16% vs 85.61%, p = 0.0001) (**Figure 4B**). To investigate whether CD39$^+$ T cells have a rather tolerance inducing role in the gut environment, we next looked for IL-10 production of these cells (33–36). Notably, CD39$^+$ CD4$^+$ as well as CD39$^+$ γδ$^+$ T cells of IBD patients showed a higher frequency of IL-10$^+$ cells than their CD39$^-$ counterparts (CD4$^+$: 3.988% vs 1.309%, p = 0.0102; γδ$^+$: 2.847% vs 0.821%, p = 0.023) (**Figure 4C**). Taken together, CD4$^+$ T cells seem to maintain their IL-17^{high}IFN-γ^{low} phenotype when migrating from peripheral blood into the gut mucosa. In contrast, CD39$^+$ γδ$^+$ T cells from peripheral blood display an IL-17A^{high}IFN-γ^{high} phenotype which is different from the IL-17A^{low}IFN-γ^{low} phenotype displayed by CD39$^+$ γδ$^+$ LPL. Gut-derived CD39$^+$ γδ$^+$ T cells were furthermore able to produce IL-10 in samples from healthy donors and patients with IBD.

The Majority of CD8$^+$ and γδ$^+$ LPL Display a Tissue-Resident Memory Phenotype That Is Associated With High Expression of CD39

Taking the major differences in CD39 expression between blood and gut and the unique cytokine profile of gut-resident CD39$^+$ γδ$^+$ T cells into account, we asked how the aforementioned CD39$^+$ T-cell populations might actually represent Trm. These Trm were effector memory cells that we identified via CD69 and CD103 expression (for gating strategy, see **Supplementary Figure S1B**). As described before by Mackay et al. (37), we found significantly higher frequencies of CD69$^+$CD103$^+$ Trm among CD8$^+$ and γδ$^+$ T cells than among CD4$^+$ T cells in the intestinal lamina propria of healthy individuals (**Figure 5A**). We compared the frequency of CD39$^+$ cells among CD69$^+$CD103$^+$ Trm with the non-Trm population (cells expressing none or only one of the markers, **Supplementary Figure S1B**). The frequency of CD39$^+$ cells was significantly higher among Trm in all subsets (γδ$^+$: 85.7% vs 25.86%, p = 0.0117; CD4$^+$: 30.37% vs 11.05%, p = 0.0078; CD8$^+$: 69.06% vs 31.52%, p = 0.0117) (**Figure 5A**).

CD39 as Trm Marker of γδ$^+$ T Cells

In addition to the elevated frequency of CD39$^+$ cells within Trm, we found a positive correlation between the well-established tissue residency marker CD69 (24, 38) and CD39 on γδ$^+$ T cells (p = 0.043, R^2 = 0.464) (**Figure 5B**). To confirm our hypothesis of the existence of this tissue-resident γδ$^+$ T-cell population, we performed a t-SNE analysis. As shown in **Figure 6**, three clusters representing γδ$^+$ T cells could be readily identified based on their expression of the γδ T-cell receptor. One cluster corresponded to Vδ2$^+$ γδ$^+$ T cells while Vδ2$^-$ γδ$^+$ T cells were divided into two clusters. The distribution of CD8$^+$ and CD4$^+$ T cells was more heterogeneous (data not shown). All three clusters of γδ$^+$ T cells expressed CD39, CD69, CD103, and CD49a, while they were negative for CD73.

Next, we evaluated whether the frequency of Trm and their CD39 expression in LPL of IBD patients was different from healthy donors. Interestingly, we were not able to detect significant differences regarding the frequency of Trm and CD39$^+$ Trm either for CD4$^+$, CD8$^+$, or γδ$^+$ T cells (**Supplementary Figure S4B**). However, we observed a trend towards a lower frequency of γδ$^+$ Trm and CD39$^+$ γδ$^+$ Trm in IBD patients compared to healthy donors (γδ$^+$ Trm: HD 84.5% vs IBD 67.64%; p = 0.2089; CD39$^+$ γδ$^+$ Trm: HD 83.94% vs

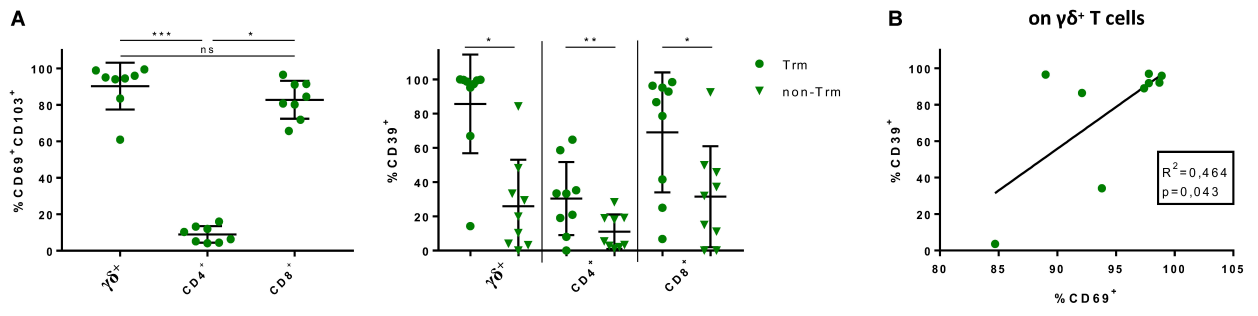

FIGURE 5 | Frequencies of CD69$^+$ CD103$^+$ γδ$^+$, CD4$^+$ and CD8$^+$ LPL; CD39 as possible signature marker for Trm. **(A)** (Left) Frequencies of CD69$^+$ CD103$^+$ γδ$^+$, CD4$^+$, and CD8$^+$ LPL from healthy donors, two data points were removed after testing for outliers; (right) Comparison of CD39$^+$ γδ$^+$, CD4$^+$, and CD8$^+$ Trm (round shapes) and non-Trm (triangular shapes) from healthy donors. **(B)** Correlation between frequency of CD69$^+$ and CD39$^+$ γδ$^+$ LPL from healthy donors. R^2 denotes Pearson's coefficient and p-value. Data of LPL of healthy donors (n = 8/9), presented as means +/- standard deviation. ns ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as calculated by Friedman test with Dunn's multiple comparisons test (**A**, left graph), Wilcoxon matched-pairs signed rank test (**A**, right graph) and Pearson's correlation analysis **(B)**. Trm, tissue-resident memory cells; LPL, lamina propria lymphocytes.

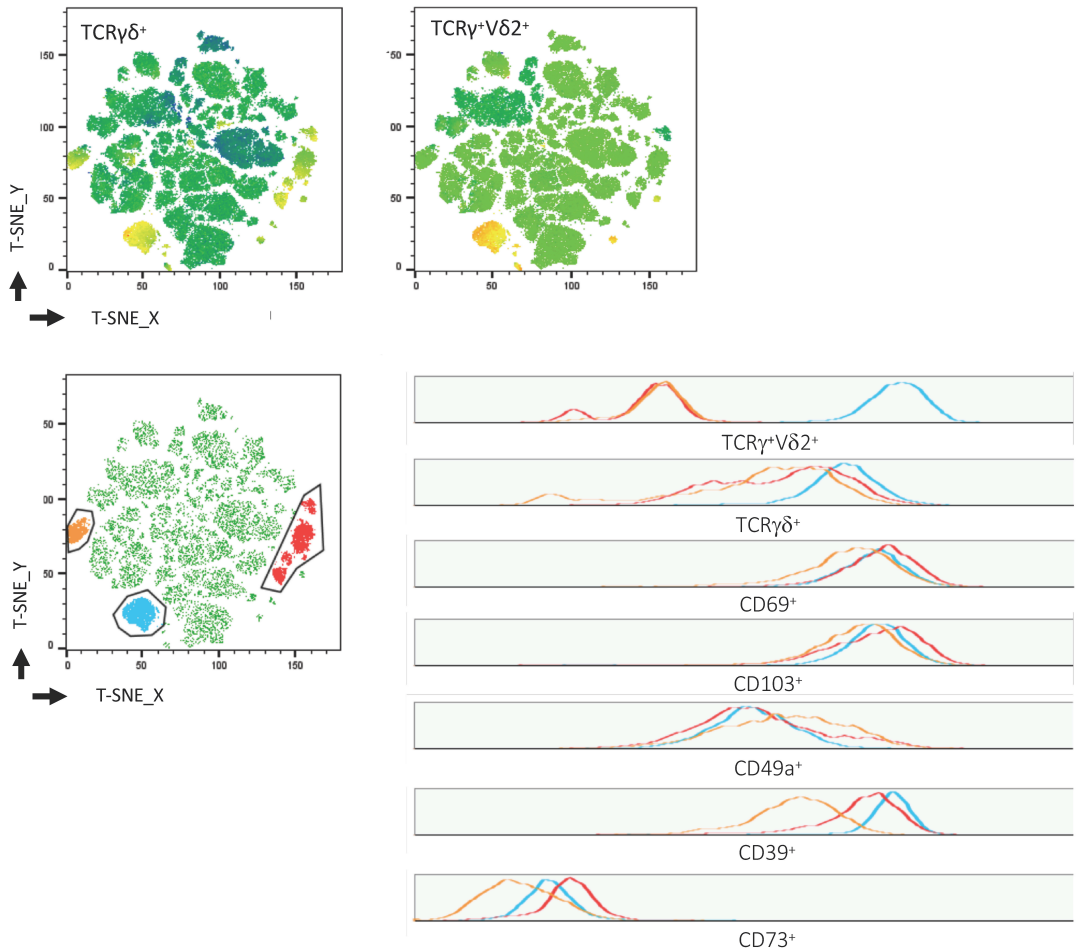

FIGURE 6 | Visualization of $\gamma\delta^+$ Trm among $CD3^+$ LPL. T-SNE map created from a concatenated file of $CD3^+$ LPL of healthy donors (n = 7). Clusters of $TCR\gamma\delta^+$ and $TCR\gamma^+V\delta2^+$ cells **(top)**. $TCR\gamma^+V\delta2^+$ (blue) and $TCR\gamma^+V\delta2^-$ (red and orange) were manually gated and overlaid on total $CD3^+$ cells **(bottom, left)**. Expression of $TCR\gamma\delta^+$, $TCR\gamma^+V\delta2^+$, CD69, CD103, CD49a, CD39, CD73 within the gated populations is depicted in histograms **(bottom, right)**. Trm, tissue-resident memory cells; LPL, lamina propria lymphocytes.

IBD 73.21%; p = 0.7955) (**Supplementary Figure S4B**). We then compared cytokine production between Trm and non-Trm in patients and healthy controls. We found higher frequencies of IL-$17A^+$ cells among the $CD4^+$ Trm compared to the $CD4^+$ non-Trm subset. This difference became significant in samples from IBD patients (**Figure 7A**). $CD8^+$ Trm also showed significantly higher frequencies of IL-$17A^+$ cells compared to $CD8^+$ non-Trm in the context of IBD. This difference was not apparent in healthy donors. We did not observe significant differences in IFN-γ production between $CD4^+$ Trm and non-Trm either in controls or patients. The frequency of IFN-γ^+ $CD8^+$ Trm from lamina propria of healthy controls was significantly lower compared to non-Trm. Remarkably, $\gamma\delta^+$ Trm displayed significantly lower frequencies of IL-$17A^+$ and IFN-γ^+ cells compared to their non-Trm counterparts in both healthy donors and IBD patients (healthy: $\gamma\delta^+$ $IL17A^+$: 0.709% vs 4.051%, p = 0.0078; $\gamma\delta^+$ IFN-γ^+: 37.95% vs 83.85%, p = 0.002; IBD: $\gamma\delta^+$ $IL17A^+$: 1.14% vs 4.799%, p = 0.0469; $\gamma\delta^+$ IFN-γ^+: 38.37% vs 76.83%, p = 0.0078) (**Figure 7B**). Taken together, the IL-$17A^{low}$IFN-γ^{low} phenotype of $CD39^+$ $\gamma\delta^+$ T cells is in line with the IL-$17A^{low}$IFN-γ^{low} phenotype of $\gamma\delta^+$ Trm. Altogether, these results support the notion that CD39 can be used as Trm marker - especially for $\gamma\delta^+$ T cells.

DISCUSSION

It is commonly agreed that the immune response in the mucosal compartment is profoundly shaped by extracellular signaling of ATP and adenosine (10, 11, 39). Several gut-derived cell populations can modulate their CD39 expression and thereby influence ATP/adenosine levels (36, 40, 41). We found a highly significant difference of the CD39 and CD73 expression of $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells between LPL and PBMC highlighting the peculiarity of the mucosal compartment where T cells adapt their phenotype and their effector functions to this special environment (21, 22, 42). In particular, peripheral $CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells displayed a $CD39^{low}CD73^{high}$ phenotype

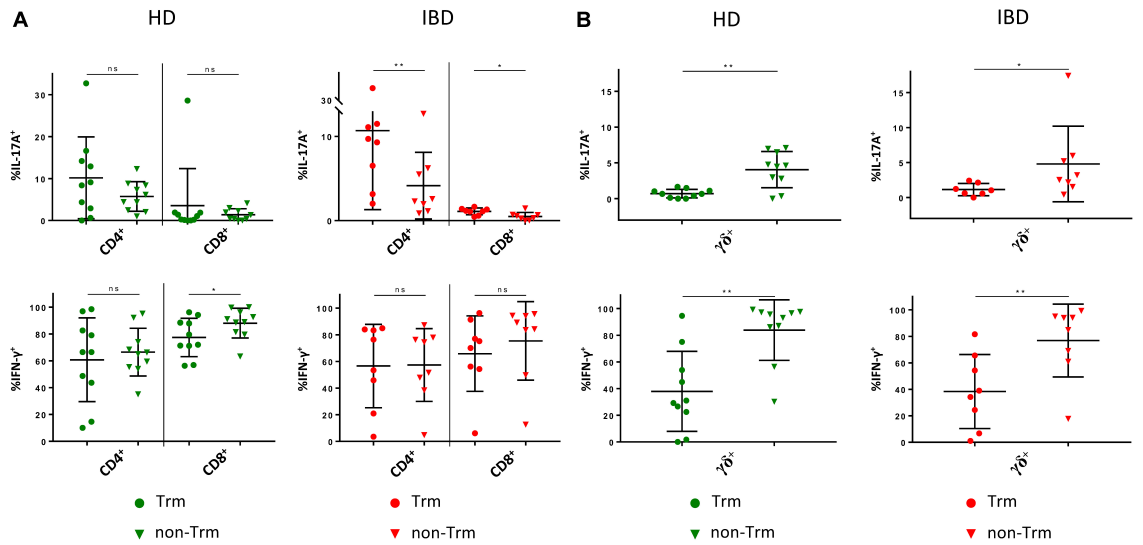

FIGURE 7 | Cytokine production by tissue-resident versus non-tissue-resident CD4$^+$, CD8$^+$, and γδ$^+$ LPL. **(A)** Production of IL-17A and IFN-γ by Trm (round shapes) versus non-Trm (triangular shapes) CD4$^+$ and CD8$^+$ LPL from healthy donors (green) and IBD patients (red). **(B)** Production of IL-17A and IFN-γ by Trm (round shapes) versus non-Trm (triangular shapes) γδ$^+$ LPL from healthy donors (green) and IBD patients (red). Data from LPL of healthy donors (n = 10) and IBD patients (n = 8), presented as means +/- standard deviation. ns ≥ 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, as calculated by Wilcoxon matched-pairs signed rank test. LPL, lamina propria lymphocytes; HD, healthy donor; IBD, patient with inflammatory bowel disease; Trm, tissue-resident memory cells.

compared to gut-derived CD8$^+$ and γδ$^+$ T cells which showed a CD39^{high}CD73^{low} phenotype.

Further details about the regulation of CD39 expression on T cells in humans need to be understood: The change of phenotype and increased expression of CD39 in the gut suggests a general association with T-cell activation (13), but increased hypoxia levels in the mucosal tissue also seem to be involved (43, 44). Hypoxia leads to elevated levels of the transcription factors HIF-1α and Sp1 which downstream lead to the upregulation of CD39 surface expression (45).

Surprisingly, we only identified small differences in the expression patterns of CD39 and CD73 between healthy controls and IBD patients. It will be important to study whether greater differences in terms of frequency, phenotype or function will be evident when analyzing larger cohorts of untreated patients with more severe IBD disease activity. Additionally, it would be interesting to distinguish between treatment groups and patients with UC or CD.

A key finding of our study was the decreased frequency of gut-derived CD39$^+$ γδ$^+$ T cells in IBD patients regardless of disease severity compared to healthy donors. Since most of the gut-resident γδ$^+$ LPL did not express the Vδ2 chain of the γδ T-cell receptor, we postulate that our observations are applicable to Vδ1$^+$ γδ$^+$ T cells as they are the dominant γδ$^+$ T-cell subset in the gut (46). We assume that the reduced frequency of CD39$^+$ γδ$^+$ T cells could indeed be crucial for the development of IBD. In a murine model of DSS-induced colitis, it was previously shown that elevated, extracellular ATP levels are associated with progression of disease (47). Consistent with a regulatory function of CD39$^+$ cells (34, 36, 48, 49), Otsuka et al. recently described a distinct subset of γδ$^+$ T cells in mice that was characterized by CD39 expression and sufficiently suppressed proliferation of and cytokine production by effector T cells (33). Thus, enzymatic activity and degradation of ATP of CD39$^+$ γδ$^+$ T cells might promote an inhibitory environment and should be monitored in future experiments.

Regulatory T cells (Tregs, CD4$^+$ CD25$^+$ FOXP3$^+$) have been described to play an important role suppressing inflammation in IBD (50–52). However, there are contradicting reports about their relative frequency in the gut (52–55) and only little is known about their interaction with γδ$^+$ T cells (56, 57). In a small subset of patients, we analyzed gut derived Tregs alongside with γδ$^+$ T cells. Neither the frequencies of Tregs in tissue of IBD patients compared to healthy donors nor CD39 expression significantly differed in this small sample size (**Supplementary Figure S6A**). However, when we performed a correlation analysis to see how Treg and CD39$^+$ γδ$^+$ T cell frequencies would correspond to each other, we found a negative association in IBD patients but not in healthy controls (**Supplementary Figure S6B**). This could indicate that an increase of Tregs in the mucosal department might be a compensatory mechanism to counteract the decrease of CD39$^+$ γδ$^+$ T cells. To better integrate this observation in the immune landscape of regulatory T cell subsets and to validate the overall frequencies of Tregs and their CD39 expression in the gut, prospective, more detailed studies with larger cohorts need to be performed.

Importantly, in IBD patients CD39$^+$ γδ$^+$ LPL displayed only low *ex vivo* FOXP3 expression but the frequency of FOXP3$^+$ CD39$^+$ γδ$^+$ LPL was significantly higher compared to healthy controls (p = 0.0221), (**Supplementary Figure S6C**).

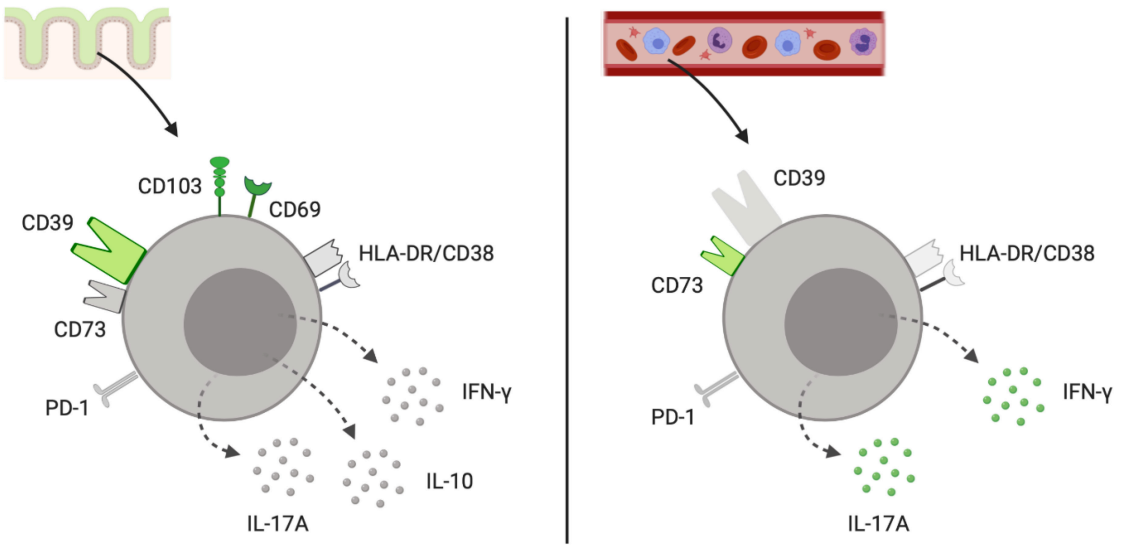

FIGURE 8 | Schematic representation of the phenotype and functional profile of tissue-resident CD39$^+$ γδ$^+$ LPL and peripheral CD39$^+$ γδ$^+$ T cells. Surface markers and intracellular cytokines colored in grey (low frequency) and green (high frequency) for CD39$^+$ γδ$^+$ Trm of the large intestine **(left)** and CD39$^+$ γδ$^+$ T cells from peripheral blood **(right)**. Illustration created with the online software BioRender (San Francisco, CA, United States). Trm, tissue-resident memory cells.

In future follow up studies it will be interesting to elucidate the detailed molecular signature of CD39$^+$ γδ$^+$ LPL with respect to the expression of other regulatory molecules and transcription factors.

In our *in vitro* experiments, the CD39$^+$ γδ$^+$ T cells exhibited an IL-17A^{low}IFN-γ^{low} phenotype in contrast to the other CD39$^+$ T-cell subpopulations in the gut. Furthermore, we found that CD39$^+$ γδ$^+$ T cells were able to produce IL-10 upon stimulation. The frequencies of IL-10$^+$ CD39$^+$ γδ$^+$ were lower than of IL-10$^+$ CD39$^+$ CD4$^+$ T cells but significantly higher compared to their IL-10$^+$ CD39$^-$ γδ$^+$ counterparts. The main findings regarding phenotypic and functional properties of CD39$^+$ γδ$^+$ LPL in contrast to peripheral CD39$^+$ γδ$^+$ are summarized in **Figure 8**. One hypothesis that needs to be further explored is whether the loss of CD39$^+$ γδ$^+$ T cells that are able to produce IL-10 and upregulate regulatory transcriptional factors like FOXP3 in the gut mucosa of IBD patients plays an important role in the onset and perpetuation of IBD.

Lymphocytes in the gut mucosa are constantly exposed to a special environment that features both commensal bacteria and potential pathogens (58). Hence, a newly defined Trm population for first-line defense is present which we hypothesize is playing a key role in modulating the immune homeostasis of the large intestine. The important Trm marker CD69 is commonly used as an early activation marker (59), but it was shown that CD69 expression is not associated with recent activation in mucosal tissue (24). Of note, CD69 expressed on LPL interacts with the Sphingosine-1-phosphate-receptor-1 and therefore prevents tissue egress (60, 61). Synergistically, the integrin CD103 binds to E-cadherin which is highly expressed on epithelial cells (62). As a consequence, the expression of CD69 and CD103 prevents T cells from recirculation between tissue and blood and their surface expression can be used to identify Trm in the intestine (23, 63). In line with Zundler et al. who described increased frequencies of CD4$^+$ Trm in LPL of IBD patients compared to healthy donors (64), we observed an accumulation of CD4$^+$ Trm in the mucosa from patients with IBD. Moreover, we detected a slightly decreased frequency of γδ$^+$ Trm in IBD. We furthermore established a link between CD39 expression and γδ$^+$ Trm, indicating the decreased frequency of CD39$^+$ γδ$^+$ LPL in IBD mirrors the loss of γδ$^+$ Trm. So far, this loss has been shown for intraepithelial CD39$^+$ γδ$^+$ and CD8$^+$ lymphocytes (19, 20). Our results confirm and extend these findings to the lamina propria of the large intestine in healthy individuals and patients with IBD.

Other studies postulate that rather than immunosuppressive activity, the interaction of Trm with dendritic cells is crucial for protection of the epithelial tissue in IBD (19). In contrast, data of Trm promoting inflammation have been published (64, 65). To our knowledge, our data are the first indicating that CD39$^+$ γδ$^+$ Trm might play a central, tolerance modulating role in the gut mucosa since significantly lower frequencies of Trm produce IFN-γ and IL-17A compared to non-Trm. Their impaired frequency in IBD patients strongly suggests that they are involved in the pathogenesis of this disease. Future studies should focus on investigating the direct immunosuppressive activity of CD39$^+$ γδ$^+$ Trm by *in vitro* inhibition assays.

However, several limitations of the current study should be noted that are inherent to heterogeneous, cross-sectionally investigated cohorts of patients with CD, UC, and IC. Patients were under different disease-specific treatments and some suffered from concomitant diseases like primary sclerosing cholangitis or autoimmune hepatitis. Additionally, three patients had a transplanted liver and received further immunosuppressive treatment. However, further analysis of the transplanted versus

not-transplanted IBD patients in this cohort did not reveal significant differences (data not shown). Future studies should be of a prospective design with longitudinal analysis of patients before and under therapy with matched blood and gut samples. Furthermore, it would be interesting how our findings are affected by CD39-encoding ENTPD1 polymorphisms (17).

Our data also seem to support the investigation of future therapeutic approaches that aim to alter purinergic signaling cascades in IBD patients in order to dampen the overall inflammation (66). Treatment suggestions based on murine models include apyrase substitution (47), increase of the extracellular adenosine concentration via mucosa-specific inhibition of adenosine uptake (67), or HIF-1α stabilization (43, 68, 69). The role of CD39-expressing, tissue-resident $\gamma\delta^+$ LPL should be highlighted and considered for prospective investigations in the treatment of IBD.

In summary, our data give a first comprehensive portrayal of CD39 and CD73 expression patterns on different T-cell populations with and without tissue-resident memory phenotype in the large intestine and peripheral blood of healthy individuals and in the context of IBD.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the Ärztekammer Hamburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JS and JL designed the study and wrote the first draft of manuscript. JS gave funding. JL, MW, and RW conducted the experiments. MK, DR, JH, and JS obtained biopsies from patients undergoing colonoscopy. JL analyzed the data. JME and MW contributed to the interpretation of the multicolour flow cytometry panels. JL prepared the figures and got input from JS, MW, and all other authors. All authors reviewed the manuscript and gave important input.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.567472/full#supplementary-material

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Control of Gut Inflammation by Modulation of Purinergic Signaling

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Inflammatory bowel disease (IBD) is a serious inflammatory condition of the gastrointestinal tract. Crohn's disease (CD) and ulcerative colitis (UC) are two of the most common IBD manifestations and are both associated with unfettered inflammation, often refractory to conventional immunosuppressive treatment. In both conditions, imbalance between effector and regulatory cell immune responses has been documented and is thought to contribute to disease pathogenesis. Purinergic signaling is a known modulator of systemic and local inflammation and growing evidences point to extracellular ATP/adenosine imbalance as a key determinant factor in IBD-associated immune dysregulation. *In vitro* and pre-clinical studies suggest a role for both ATP (P2) and adenosine (P1) receptors in dictating onset and severity of the disease. Moreover, our experimental data indicate ENTPD1/CD39 and CD73 ectoenzymes as pivotal modulators of intestinal inflammation, with clear translational importance. Here we will provide an updated overview of the current knowledge on the role of the purinergic signaling in modulating immune responses in IBD. We will also review and discuss the most promising findings supporting the use of purinergic-based therapies to correct immune dysregulation in CD and UC.

Keywords: adenosine receptor, P2 receptor, ectonucleotidase, crohn's disease, ulcerative colitis

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INTRODUCTION

Healthy tissues contain negligible levels of extracellular nucleotides and nucleosides; whereas inflammatory sites are characterized by accumulation of extracellular ATP and adenosine.

Release of nucleotides in the extracellular environment triggers P2 receptors activation on target cells. The P2 receptor family includes seven P2X and eight P2Y members, classified based on their desensitization time and affinity for the ligand. P2 receptors are virtually present on all immune cells, are mainly activated by extracellular ATP and have been generally described as mediators of inflammatory processes (1).

Once released in the extracellular environment, nucleotides can be rapidly hydrolyzed into nucleosides by specific ectonucleotidases. Ectonucleotidases are expressed on the surface of different immune cell types and belong to several enzymatic families, which have been functionally and structurally characterized (2, 3). The prototype member of the NTPDase family is ENTPD1/CD39, a rate-limiting ectoenzyme that hydrolyzes ATP into AMP, which is then further degraded into adenosine by the ecto-5′-nucleotidase/CD73 (2).

Once generated, extracellular adenosine can activate P1 receptors (adenosine receptors) on target cells. Adenosine receptors are classified into four subtypes (A1, A2A, A2B, and A3) and consist of G-coupled, 7-transmembrane spanning receptors expressed by a wide range of immune cells. Adenosine receptors have been mainly associated with immunoregulatory functions (4). **Figure 1** shows how the purinergic signaling modulates immune responses during inflammation.

In this review, we will discuss the role of the purinergic signaling, with a focus on P1 and P2 receptors and on ENTPD1/CD39 and CD73 ectoenzymes, in the context of inflammatory bowel disease (IBD). We will report the most important findings obtained from human studies and experimental models of colitis, and we will also highlight potential novel therapeutic approaches, such as administration of exogenous recombinant ectonucleotidases and targeting of specific intracellular pathways that interfere with purinergic signaling.

IBD is a chronic inflammatory condition of the gastrointestinal tract associated with altered gut microbial composition, disrupted mucosa structure and systemic biochemical alterations (5, 6). IBD most frequent manifestations include Crohn's disease (CD) and ulcerative colitis (UC), which are diagnosed based on the localization of intestinal inflammation and clinical symptoms (5, 7).

Mounting evidence has indicated Th17-cells as the main effector players involved in IBD tissue damage and several additional studies have reported the role of purinergic signaling alterations in the immunopathogenesis of the disease.

Recommended standard therapies for IBD include corticosteroids (7), immunosuppressive drugs (8, 9), aminosalicylates (10, 11), and biological agents like infliximab (12). These currently available treatments, however, are often associated with adverse effects and limited therapeutic efficacy, this emphasizing the need for novel and more effective therapies. Given the role played in the modulation of immune responses, the purinergic signaling might represent a potential therapeutic target.

P2X RECEPTOR FAMILY MEMBERS - P2X7

Alterations of purinergic signaling play an important role in promoting tissue inflammation in IBD and several evidences support the involvement of P2X receptors and specifically the P2X7 receptor (P2X7R).

Systemic P2X7R inhibition by administration of the selective inhibitor A740003 or brilliant blue G, prevents the development of TNBS-induced colitis in rat models (13). Similarly, P2X7R deletion is protective in murine models of colitis (14).

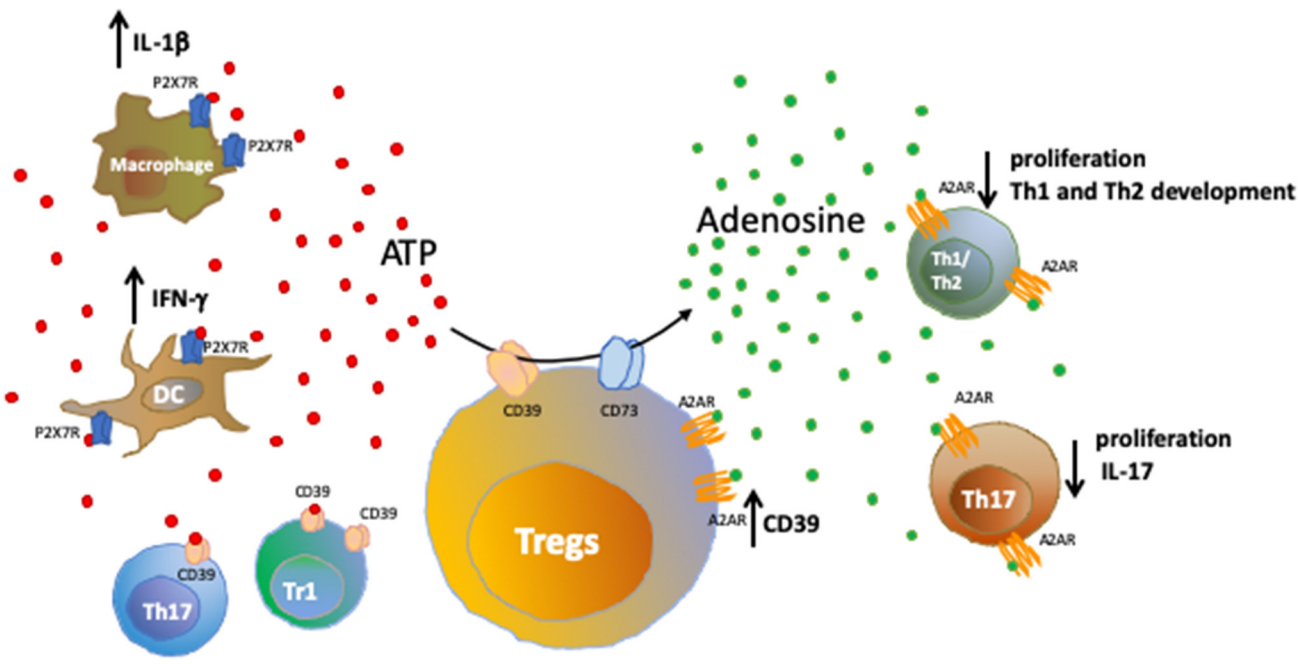

FIGURE 1 | Purinergic signaling in immune cells. Healthy tissues contain negligible levels of extracellular nucleotides; whereas inflammatory sites are characterized by accumulation of extracellular ATP that binds purinergic receptors on target cells, further amplifying inflammatory responses. We show here the effects resulting from the activation of ATP-P2X7R axis in macrophages and dendritic cells. Extracellular nucleotides can be converted into nucleosides by ectonucleotidases present on Tregs, T regulatory type-1 (Tr1) cells and a subset of Th17-cells. The activity of CD39 and CD73 ectoenzymes expressed by Tregs and converting pro-inflammatory ATP into anti-inflammatory adenosine is shown. Upon binding to the A2A receptor (A2AR), extracellular adenosine suppresses effector T cell responses, leading to reduced cell proliferation, limited Th1 and Th2 development and control of IL-17 production by Th17-cells. Notably, A2AR is expressed also by Tregs and its activation promotes CD39 expression.

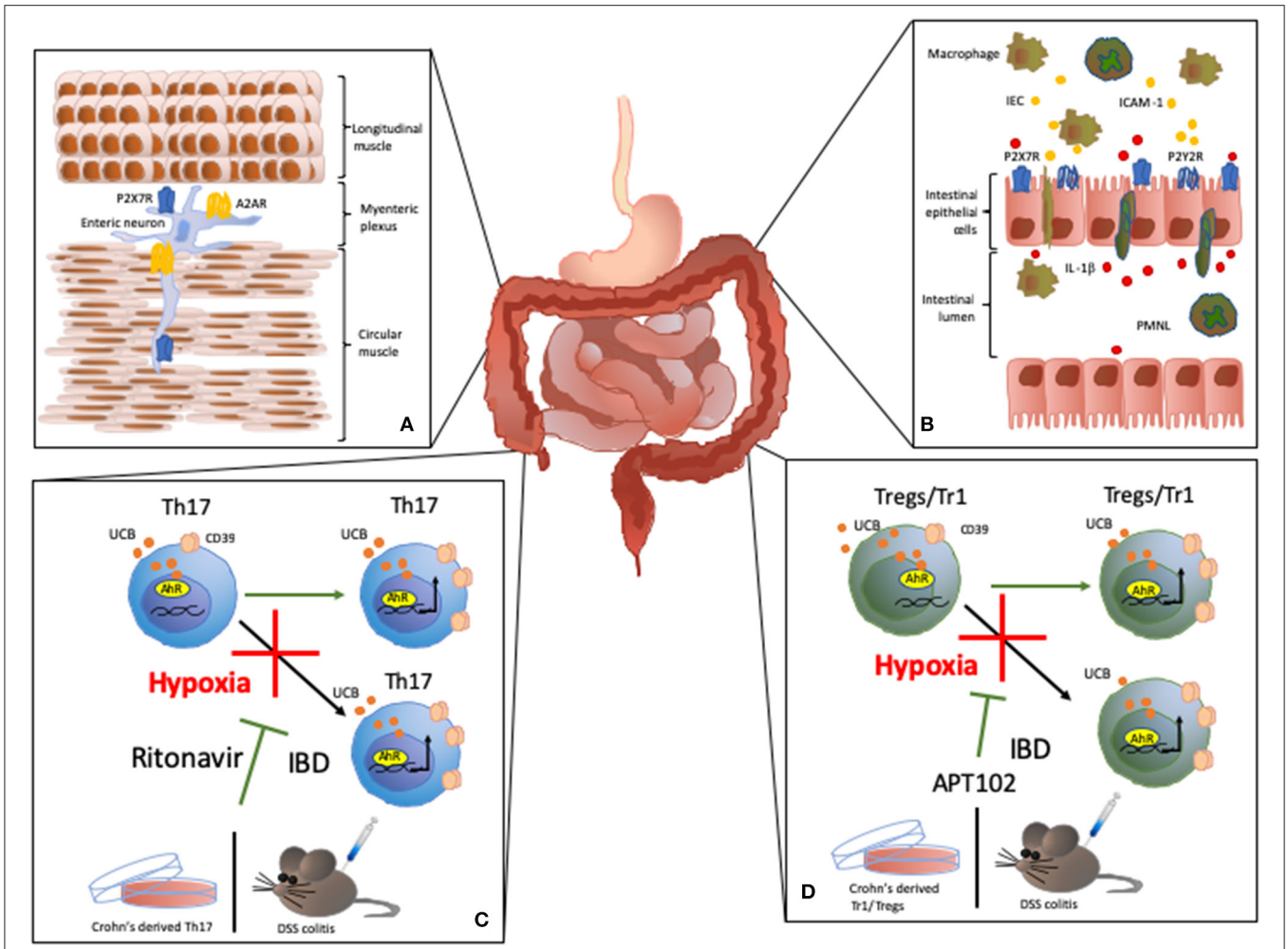

FIGURE 2 | Effects of purinergic signaling modulation in IBD. **(A)** Effects on colon motility: P2X7R activity has been associated with death of enteric neurons that leads to impaired colon motility in IBD (15). A2AR activity modulates the inhibitory effect of adenosine on colonic motility during experimental colitis. **(B)** P2Y receptor (P2Y2R) expression and activation on intestinal epithelial cells (IEC) increases the expression of chemoattractive proteins, like ICAM-1, in IEC, promoting macrophage transepithelial migration and adhesion (16). In the inflamed lamina propria and gut epithelium, P2X7R activation triggers IL-1β secretion by IEC in response to polymorphonuclear leukocyte (PMNL) transmigration (17). **(C,D)** In Tr1, Treg, and Th17-cells, CD39 expression is induced upon activation of aryl hydrocarbon receptor (AhR), an intracellular receptor for pollutants, toxins and endogenous compounds, like unconjugated bilirubin (UCB). UCB boosts cell immunoregulatory properties *in vitro* and ameliorates the course of experimental colitis *in vivo* (18). However, in Crohn's disease or experimental colitis, Treg, Tr1, and Th17-cells are refractory to the regulatory effects of UCB due to a deleterious effect of hypoxia (19). Notably, we found that HIF-1α inhibition or ABC transporters blockade obtained upon administration of the antiretroviral ritonavir, limits the detrimental effects of hypoxia in Th17-cells *in vitro* in *in vivo* (19). Similarly, administration of the ADPase APT102, restores the response of Treg and Tr1-cells to the regulatory effects of UCB and ameliorates experimental colitis *in vivo* (20).

P2X7R activation has been also associated with the death of enteric neurons that lead to impaired colon motility (**Figure 2A**). In rat models of ulcerative colitis, P2X7R co-localizes with immunoreactive cells in the myenteric plexus (15). The decrease in neuronal density in the myenteric plexus has a positive correlation with P2X7R expression in different neuronal populations present in the area (15). P2X7R stimulation triggers pannexin-1 (Panx1) channels opening and inflammasome activation, including the Asc adaptor protein and caspase cleavage, which results in neuronal death. Accordingly, in murine models of colitis, selective inhibition of each component of the P2X7R-inflammasome axis significantly dampens the inflammation while Panx1 inhibition reduces the colonic dysfunction *in vivo* (21).

Additional evidence has indicated that in a model of 2,4-dinitrobenzenesulfonic-acid (DNBS)-induced colitis P2X7R is upregulated in the neuromuscular layer; notably, increase in electrically induced contractions was recorded in colonic preparations, obtained from colitic mice, and exposed to A804598, a selective P2X7R antagonist (22).

P2X7R expression promotes intestinal inflammation also by inducing different subsets of effector cells, including mast cells. Increased numbers of mast cells were found in the colon of CD patients and experimental murine models of colitis (23). Mast

cell-deficient or P2X7R$^{-/-}$ mast cell-reconstituted mice present lower susceptibility to inflammation as compared to the wild type counterpart. Interestingly, P2X7R-mediated activation of mast cells triggers release of pro-inflammatory cytokines, chemokines, and leukotrienes that recruit neutrophils in the inflamed area (23). Further, P2X7R activation has been linked to death and retention of regulatory T-cells (Tregs) in the mesenteric lymph nodes impairing gut immune tolerance (14).

The inflammatory process in the gut is linked to polymorphonuclear leukocyte (PMNL) transmigration into the mucosa leading to increased IL-1β production by intestinal epithelial cells (IEC). Interestingly, in CD patients, P2X7R is overexpressed in the inflamed lamina propria and gut epithelium, where P2X7R activation triggers IL-1β secretion by IEC in response to transmigration of PMNL (17) **(Figure 2B)**.

In the intestinal mucosa, P2X7R is also present on macrophages and dendritic cells where the expression of this receptor positively correlates with IFN-γ, TNF-α, and IL-1-β levels, this leading to epithelial cells apoptosis. Concomitantly, lower concentrations of IL-10 have been also detected (24). *In vitro* experiments, conducted on human colonic mucosa strips, have revealed that blockade of Panx1 and P2X7R significantly reduce crypt damage, pro-inflammatory cytokine release, loss of tight junctions, and cell permeability (25).

It has been hypothesized that the presence of a gain of function single nucleotide polymorphism or the loss of function SNPs (His155Tyr, Arg307Gln, and Glu496Ala) affect P2X7R activity and could be associated with susceptibility to CD. However, no significant differences were noted among the subjects carrying the polymorphisms in terms of disease incidence (26). A phase II clinical trial on patients with moderate to severe CD evaluated the use of a selective P2X7R antagonist, AZD9056, as a potential therapeutic approach. Despite not having effects on the levels of inflammatory biomarkers, oral administration of AZD9056 induced an overall improvement in the disease symptoms (27). Further investigations are therefore needed to develop P2X7R antagonists that effectively interfere with the inflammatory response; in this regard, several compounds have been proposed and these include Pyroglutamide-Based P2X7R Antagonists (28).

P2X7R, however, plays also an important role in the regulation of follicular T helper cell density in Peyer's patches (29) while favoring the generation of metabolic homeostasis by sensing microbiota-derived ATP (30).

P2Y RECEPTORS

The G-coupled associated P2Y receptors (P2YR) are also involved in IBD immunopathogenesis. *In vitro* experiments using human nerve-gut preparations and mouse colonic sensory neurons, showed that P2YR activation triggers visceral nociceptors stimulation that can lead to the visceral pain associated with inflammation (31).

Colonic tissue isolated either from IBD patients or mice with experimental colitis displays higher expression of P2Y2 receptor (P2Y2R) when compared to healthy controls. Further investigations have revealed that increase in P2Y2R expression depends on the activity of the C/EBPβ transcription factor, which is also upregulated in IEC in murine models of colitis (32). Investigations conducted on human colon cell lines and colonic tissue from CD and UC patients, revealed that during intestinal inflammation P2YR expression is also regulated through a NF-kB p65-dependent mechanism (33). Activation of P2YR, and particularly P2Y2R, increases the expression of chemoattractive proteins, like ICAM-1, in IEC; this promoting macrophage transepithelial migration and adhesion (16) (**Figure 2B**).

On the other hand, experiments conducted in murine models of dextran sulfate sodium (DSS) colitis showed that administration of the P2Y2R agonist 2-thioUTP reduces the disease activity index and histological scores. These evidences suggest a role for P2Y2R in the remission phase of IBD (34).

Another study identified a specific activity of P2Y6R that was found to regulate CXCL8 expression in IEC, promoting neutrophil recruitment and inflammatory responses (35). In contrast, in murine models of DSS colitis, P2Y6R deletion has been associated with extensive intestinal inflammation, resulting from increased recruitment of Th17/Th1 lymphocytes in the gut mucosa (36).

In humans, P2Y6R is expressed in a wide range of inflammatory cells and expression levels increase in activated CD4$^+$ and CD8$^+$ T-cells. Because of the pro-inflammatory activity, P2Y6R has been implicated in the pathogenesis of IBD-mediated intestinal damage (37).

ENTPD1/CD39 AND CD73 ECTOENZYMES

The involvement of ENTPD1/CD39 and CD73 ectoenzymes in the modulation of intestinal inflammation has been extensively studied.

In experimental mouse models of colitis, the impact of CD39 expression strictly depends on the model considered as well as on the cell populations involved in the tissue damage. In the setting of trinitro-benzene-sulfonic-acid (TNBS)-induced colitis in humanized mice, Goettel et al., demonstrated that activation of the transcription factor aryl hydrocarbon receptor (AhR)—an intracellular receptor for pollutants, toxins, and endogenous compounds—by indole-3′-carbonyl-thiazole-4-carboxylic-acid-methyl-ester, induced Tregs and this was linked to CD39 upregulation (38). In contrast, a more favorable course of TNBS-induced colitis has been observed in CD39-null mice when compared to wild type controls (39). In the context of DSS colitis, ENTPD1/CD39 and CD73 expression on activated macrophages was found to limit inflammation, either by directly hydrolyzing pro-inflammatory extracellular ATP into adenosine or by indirectly promoting Treg development (40). Further, in the same experimental model, CD39 deletion exacerbated colitis as reflected by heightened disease activity index, higher levels of pro-inflammatory markers and histological evidence of tissue injury.

Notably, in humans, the presence of SNPs associated with lower levels of CD39 expression correlate with increased susceptibility to CD (41); whereas increased CD39 levels in peripheral blood Tregs are associated with clinical and

endoscopic remission (42). Recently Huang and colleagues reported multiple cellular defects in pediatric colitis and IBD, including impaired cyclic AMP-response signaling, infiltration of phosphodiesterase 4B and TNF-expressing macrophages, platelet aggregation and decrease in CD39-expressing intraepithelial T-cells (43); notably, administration of the phosphodiesterase inhibitor dipyridamole ameliorated colitis symptoms in a pilot study (43).

CD39 is known to play an important role in the immunosuppressive activity of suppressor Th17-cells (supTh17). This cell population derives from iTregs upon exposure to Th17 polarizing conditions. SupTh17-cells display high expression of CD39 and actively contribute to the production of adenosine. Compared to *bona fide* pathogenic Th17, supTh17-cells display higher expression of the enzyme adenosine deaminase and lower expression of A2A receptor (A2AR), these features making these cells refractory to the inhibitory effects of adenosine (44).

We recently found that, in human Th17-cells, CD39 expression is induced upon activation of AhR via unconjugated bilirubin (UCB). Exposure to UCB boosts Th17 immunoregulatory properties *in vitro* and ameliorates the course of experimental DSS colitis *in vivo* (18). Furthermore, Th17-cells from CD patients are refractory to the immunosuppressive effects of UCB. This lack of response is directly dependent on high levels of hypoxia-inducible-factor-1alpha (HIF-1α) that promotes ABC transporters to favor UCB exit from the cells and therefore limiting AhR activation. Interestingly, blockade of HIF-1α or ABC transporters limits the detrimental effects of hypoxia both *in vitro* and *in vivo* (19) (**Figure 2C**). Further, human CD39 overexpression or administration of APT102—the extracellular domain with improved ADPase activity of human nucleoside triphosphate diphosphohydrolase-3 (CD39L3), a member of the CD39 family—restores AhR-mediated regulatory effects on CD-derived Tregs *in vitro* and prevents hypoxia-related damage in experimental colitis *in vivo* (20) (**Figure 2D**).

There is however evidence that in IBD patients with active disease, CD73 expression in $CD4^+$ T-cells is associated with a pro-inflammatory Th17-cell phenotype; based on this evidence, CD73 could be therefore used as a marker to monitor disease activity during treatment (45).

A2AR

In IBD, the beneficial effects of adenosine generation by CD39 and CD73 ectoenzymes, have been supported by a wealth of studies. In rat models of chronic experimental colitis, administration of two different selective adenosine deaminase inhibitors significantly improved the course of disease (46). Data revealed that both compounds significantly decreased the inflammatory parameters and the beneficial effect was abrogated in the presence of pharmacological blockade of A2AR or A3 receptor (A3R), suggesting a protective role for both these receptors (46).

A study on the effects of electroacupuncture on visceral pain in a murine model of TNBS-colitis, showed that the beneficial effect of the treatment was linked to increased expression of A1R, A2AR, and A3R and to decreased expression of A2B receptor (A2BR) in colonic tissue. The antalgic effect was mediated by inhibition of release of the pro-inflammatory factors substance P (SP) and IL-1β. This salutary effect was partially abrogated in the presence of adenosine receptors antagonists (47).

A2AR activity has been associated with amelioration of spontaneous ileitis and administration of the A2AR agonist ATL-146e significantly reduced the intestinal mucosa inflammation, leukocyte infiltration in the gut and release of proinflammatory cytokines (48). A2AR plays also an important role in modulating colonic motility. In this regard, exposure of rat colonic longitudinal muscle preparations to the receptor antagonist ZM 241385 increases transmural electrical stimulation-induced contractions, whereas exposure to the receptor agonist CGS 21680, triggered a concentration-dependent reduction of contractile responses. Interestingly, these modulatory functions are further enhanced in preparations derived from animals exposed to DNBS-induced colitis (49). Similarly, in rat ileum/jejunum preparations, CGS 21680 administration prevented the TNBS-related inhibition of acetylcholine-induced contractions and A2BR antagonist PSB-1115 inhibited the contraction-decreasing effect of TNBS. The effect was even enhanced in response to combinatorial treatment with CGS 21680 and PSB-1115, both used at subthreshold concentrations (50). The beneficial role of A2AR in the context of experimental colitis has been also supported by the observation that administration of the A2AR agonist polydeoxyribonucleotide (PDRN), ameliorated the clinical symptoms and promoted tissue repair in two models developed in Sprague-Dawley rats. PDRN administration significantly reduced the circulating levels of pro-inflammatory cytokines, along with decrease in malondialdehyde and myeloperoxidase activity (51). A newly synthetized polar A2AR agonist, in which polar groups were introduced to prevent peroral absorption and subsequent systemic side effects, has been proposed as a potential treatment for IBD. Preliminary experiments conducted in rat ileum/jejunum preparations showed a significant improvement of the impaired acetylcholine-induced contractions and this beneficial effect was boosted by A2BR selective antagonists (52). Importantly, administration of this compound in a rat model of oxazolone-induced colitis limited weight loss and decreased levels of TNF-α in colonic tissue (53).

In humans, overexpression of the miRNA-16 has been reported in UC patients. A recent study identified a correlation between miRNA-16 overexpression and A2AR downregulation in the colonic mucosa of active UC patients. In the same study it was also reported that miRNA-16 inhibits the expression of the *A2AR* gene by acting at the post-transcriptional level; this effect being mediated upon engagement of the NF-κB pathway (54).

A2BR

Despite the widely described immunoregulatory effects of adenosine, there is evidence supporting a pro-inflammatory role of A2BR in the context of intestinal inflammation.

Experiments conducted in murine models of colitis induced by DSS, TNBS, and Salmonella typhimurium showed a protective effect of A2BR knockout that was associated with lower neutrophil responses, although cell recruitment to the inflammatory site was not impacted (55). Accordingly, administration of the A2BR selective antagonist ATL-801 in DSS-treated wild type or piroxicam-treated IL-$10^{-/-}$ mice, significantly lowered severity of colitis along with levels of pro-inflammatory cytokines (56). Engagement of A2BR has been also linked to the damage associated with intestinal ischemia reperfusion (I/R) injury and hypoxia. In this regard, administration of the A2BR antagonist PSB-1115 results in protection of the intestinal epithelial structure in a murine model of intestinal I/R and in an *in vitro* model of acute hypoxia (57). Combinatorial administration of PSB-601—another A2BR antagonist—and the A2AR agonist PSB-0777 was found to limit the TNBS-induced contractile disruption in rat ileum/jejunum preparations (52). On the other hand, deletion of A2BR in IEC has been reported having a protective role (55). As an example, an epithelial-specific A2BR deletion resulted in a milder form of experimental colitis, when compared to wild type controls. Further, *in vitro* studies have shown that the receptor activation on epithelial cells enhances a specific barrier repair response by inducing phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (58).

A3R

The A3R has been also implicated in the modulation of intestinal inflammation. In this context, there have been antithetical reports, providing evidences for either a protective or pathogenic role for this receptor during intestinal inflammation.

In a murine model of experimental colitis induced by intrarectal administration of DNBS, the beneficial effect of adenosine deaminase inhibitors was abrogated in the presence of A2AR and A3R antagonists, suggesting a protective role for both receptors (46). Further, inhibition of visceral pain by electroacupuncture in mice with TNBS-induced colitis, are accompanied by upregulation of A3R along with A2AR and A1R (47). Conversely, a study conducted in a murine model of DSS-induced colitis, revealed a pathogenic effect for the A3R. The impact of A3R deletion was evaluated on the clinical course of experimental colitis and on colon motility that was assessed upon measurement of artificial bead-expulsion, stool-frequency and FITC-dextran transit (59). Interestingly, A3R deficiency protected from DSS-induced tissue damage, limiting the $CD4^{+}$-cell infiltration in the colon and preserving colon motility (59). The pathogenic role of A3R was also suggested by a clinical study that reported higher levels of A3R in PBMCs from patients with different autoimmune disorders, including CD, when compared to healthy subjects (60).

However, the effects of A3R expression strictly depend on the cells, in which the receptor is expressed. A study conducted on human colonic epithelial cells reported that A3R activation inhibits NF-kB signaling pathway leading to inhibition of IL-8 and IL-1β pro-inflammatory cytokines (61). In line with this observation, the use of the A3R agonist N(6)-(3-iodobenzyl)-adenosine-5-N-methyluronamide in a rat chronic model of TNBS-induced colitis showed beneficial effects on the course of the disease. Interestingly, the receptor inhibitor limited colitis-induced upregulation of other pro-inflammatory purinergic receptors like P2X1, P2X4, P2X7, P2Y2, P2Y6, as well as A2AR and A2BR (62).

Importance of the adenosinergic signaling has been further supported by the findings of Aherne et al. who showed that increased intestinal adenosine levels resulting from epithelial specific deletion of equilibrative nucleoside transporter 2 protected from inflammation in mice with experimental colitis (63).

Supplementary Table 1 summarizes the effects of purinergic receptors in IBD pathophysiology.

CONCLUDING REMARKS

Mounting clinical evidence and research data support the involvement of purinergic signaling alterations in IBD pathogenesis, with imbalances in the ATP/adenosine ratio being regarded as underlying immunological dysregulation in this condition. As already observed in other autoimmune conditions, promising therapeutic candidates based on adenosine or ENTPD1/CD39 and CD73 ectonucleotidases have been identified.

Boosting CD39 expression either by inducing AhR-signaling or by administering exogenous ADPase, which displays ectoenzymatic activity comparable to human CD39, showed important immunoregulatory effects *in vitro* and *in vivo*, in experimental colitis models. Encouraging pre-clinical data support also the use of selective A2AR agonists, in association with specific inhibition of A2BR. Inhibition of P2X7R-mediated responses has been also associated with beneficial effects.

Taken together, the currently available evidences, implicate the use of purinergic-mediated strategies as adjunctive treatments to correct immune dysregulation in IBD patients.

AUTHOR CONTRIBUTIONS

MV wrote the manuscript. SM helped drafting some sections of the manuscript. SR and ML reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01882/full#supplementary-material

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Mitochondria Synergize With P2 Receptors to Regulate Human T Cell Function

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Intracellular ATP is the universal energy carrier that fuels many cellular processes. However, immune cells can also release a portion of their ATP into the extracellular space. There, ATP activates purinergic receptors that mediate autocrine and paracrine signaling events needed for the initiation, modulation, and termination of cell functions. Mitochondria contribute to these processes by producing ATP that is released. Here, we summarize the synergistic interplay between mitochondria and purinergic signaling that regulates T cell functions. Specifically, we discuss how mitochondria interact with P2X1, P2X4, and P2Y11 receptors to regulate T cell metabolism, cell migration, and antigen recognition. These mitochondrial and purinergic signaling mechanisms are indispensable for host immune defense. However, they also represent an Achilles heel that can render the host susceptible to infections and inflammatory disorders. Hypoxia and mitochondrial dysfunction deflate the purinergic signaling mechanisms that regulate T cells, while inflammation and tissue damage generate excessive systemic ATP levels that distort autocrine purinergic signaling and impair T cell function. An improved understanding of the metabolic and purinergic signaling mechanisms that regulate T cells may lead to novel strategies for the diagnosis and treatment of infectious and inflammatory diseases.

Keywords: P2X4, mitochondria, inflammation, P2X1, P2Y11

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INTRODUCTION

ATP is the main energy carrier of living cells. Therefore, it came as a surprise to many when Geoffrey Burnstock first reported that neurons release a portion of their cellular ATP and that the released ATP acts as a signaling molecule for cell-to-cell communication (1). Subsequently, similar ATP signaling mechanisms were identified in many other tissues and organ systems (2, 3). Purinergic signaling enables single cells in a multicellular system to calibrate their individual responses in order to serve the collective interest of the entire organism. Purinergic signaling comprises three basic elements: (i) mechanisms that produce and release ATP into the pericellular space; (ii) purinergic receptors that recognize released ATP and its metabolites and elicit intracellular signals that regulate cell functions; (iii) mechanisms that terminate purinergic signaling by enzymatic breakdown of ATP, cellular re-uptake, or simple diffusion of ATP and its metabolites away from cells.

Intact cells can release ATP via vesicular exocytosis or ATP-permeable membrane channels that include connexin hemichannels, pannexin channels, calcium homeostasis modulator 1, maxi-anion channels, and volume-regulated anion channels (4, 5). Of these mechanisms, pannexin 1 (panx1) channels are particularly important in immune cells (6–10). Under basal conditions, resting cells

release only a small portion of their cellular ATP. However, mechanical stimuli or the ligation of cell surface receptors such as the antigen and chemokine receptors of T cells rapidly increase cellular ATP release (10–12). While regulated ATP release fine-tunes cell responses, excessive ATP leakage from dying cells or damaged tissues can act as a danger signal that exacerbates inflammation, impairs T cell functions, and disrupts immune responses (13–16).

ATP release and its breakdown products defines immune cell functions by autocrine stimulation of three different families of purinergic receptors, namely P1, P2X, and P2Y receptors. Different combinations of these receptors are present on the surfaces of virtually all mammalian cells, including the different immune cell subtypes (17). P1 receptors, which recognize adenosine, comprise four subtypes: A1, A2a, A2b, and A3 receptors. P2X receptors recognize ATP and consist of seven members (P2X1-7). Human P2Y receptors comprise eight members that recognize a wider range of ligands (18–21). P2Y2, P2Y4, P2Y11, and P2Y13 receptors are activated by ATP; but certain P2Y receptors also recognize other nucleotides including ADP (P2Y1, P2Y12, P2Y13), UTP (P2Y2, P2Y4, P2Y6), UDP (P2Y4, P2Y6), and UDP-glucose (P2Y14) (21, 22). P1 and P2Y receptors belong to the G protein-coupled receptor (GPCR) superfamily, while P2X receptors are ATP-gated cation channels that facilitate the influx of extracellular Ca^{2+}.

Purinergic receptors differ greatly in their desensitization kinetics and affinities for their individual ligands. The extracellular concentrations of these ligands depend on the activities of ectoenzymes expressed on the cell surface (23). Several different groups of these enzymes have been identified including ectonucleoside triphosphate diphosphohydrolases (ENTPDases), ectonucleotide pyrophosphatases/phosphodiesterases (ENPPs), ecto-5′-nucleotidase (CD73), adenosine deaminase (ADA), as well as alkaline phosphatases (23–25). These enzymes are widely distributed among the different immune cell subpopulations (24). CD39 (ENTPD1) that converts extracellular ATP and ADP into AMP, and CD73 that degrades AMP to adenosine are particularly important modulators of purinergic signaling in immune cells (26, 27). Once released from cells, ATP and its breakdown products can either diffuse away from cells or be internalized by equilibrative and concentrative nucleotide transporters that are embedded in the cell membrane and return ATP and its breakdown products for recycling and reuse in cell metabolism (28). The distribution patterns of ATP release sites, ectonucleotidases, and nucleoside transporters along with their relative proximity to P1 and P2 receptors are important determinants of the purinergic signaling mechanisms that regulate immune cell functions.

P2X1 RECEPTORS MAINTAIN MITOCHONDRIAL METABOLISM OF QUIESCENT T CELLS

Autocrine purinergic signaling is an important mechanism of immune cell regulation (17, 29–33). Human T cells express A2a, A2b, A3, P2X1, P2X4, P2X5, and P2X7, as well as all eight P2Y receptor subtypes (34–36). P2X1, P2X4, P2Y11, and P2X7 receptors have particularly important roles in the regulation of CD4 T cells (10–12, 36–40). Among these receptors, P2X1 receptors are most sensitive with an EC_{50} value of 50-1000 nM ATP (22, 41). Such ATP levels are well within the concentration range found in the pericellular environment of quiescent T cells (42). Constitutive ATP release from cells overexpressing P2X receptors is sufficient to sustain the modest Ca^{2+} uptake that preserves basal mitochondrial metabolism and ATP synthesis of resting cells (43). P2X1 receptors maintain mitochondrial metabolism in quiescent human CD4 T cells by facilitating cellular Ca^{2+} influx that sustains basal mitochondrial Ca^{2+} levels (44). Inhibition of mitochondrial metabolism and interruption of the electron transport chain impairs T cell migration, indicating that mitochondrial ATP production fuels the purinergic signaling mechanisms needed for immune surveillance and T cell functions (12, 45). Indeed, mitochondrial defects and T cell suppression are cardinal features of sepsis that correlate with morbidity and clinical outcome (44, 46–49). Taken together, these findings suggest that P2X1 receptor-mediated Ca^{2+} influx, mitochondrial ATP production, basal ATP release, and autocrine feedback through P2X1 receptors represent a purinergic-metabolic signaling loop that maintains cell metabolism of quiescent T cells and allows these cells to mount the responses needed for effective host immune defense following chemokine or antigen stimulation (**Figure 1A**).

P2X4 RECEPTORS AND MITOCHONDRIAL METABOLISM PROMOTE T CELL MIGRATION

Stimulation of CXCR4, CCR5, CCR7, and other chemokine receptors leads to the recruitment of T cells to lymphoid organs where cell migration enables them to engage and interact with antigen-presenting cells (APCs) (50–52). Stimulation of CXCR4 by stromal cell-derived factor 1α (SDF-1α) causes rapid surges of mitochondrial ATP synthesis and panx1-mediated ATP release from CD4 T cells (12, 53). The resulting pericellular ATP levels trigger P2X4 receptors with an estimated EC_{50} value ranging between 0.5 and 10 μM (22, 41). Autocrine stimulation of P2X4 receptors promotes waves of Ca^{2+} influx that further upregulate mitochondrial ATP synthesis to the levels needed for active T cell migration (**Figure 1B**) (12). P2X4 receptors aggregate in raft-like structures that associate with mitochondria primarily at the front of migrating T cells where localized ATP synthesis fuels pseudopod protrusion and forward movement of the cells. These P2X4 receptor-driven mechanisms are particularly critical for T cells that move slowly in order to probe their surroundings for potential antigens (12). Faster moving lymphocytes, however, gather their mitochondria primarily at the uropod where the bulk of ATP may be required to fuel actomyosin motor functions needed for rapid cell migration (45). Inhibition of mitochondrial ATP synthesis, ATP release, or P2X4 receptor signaling impairs the ability of T cells to polarize and to migrate in response to CXCR4 stimulation (12, 45, 53).

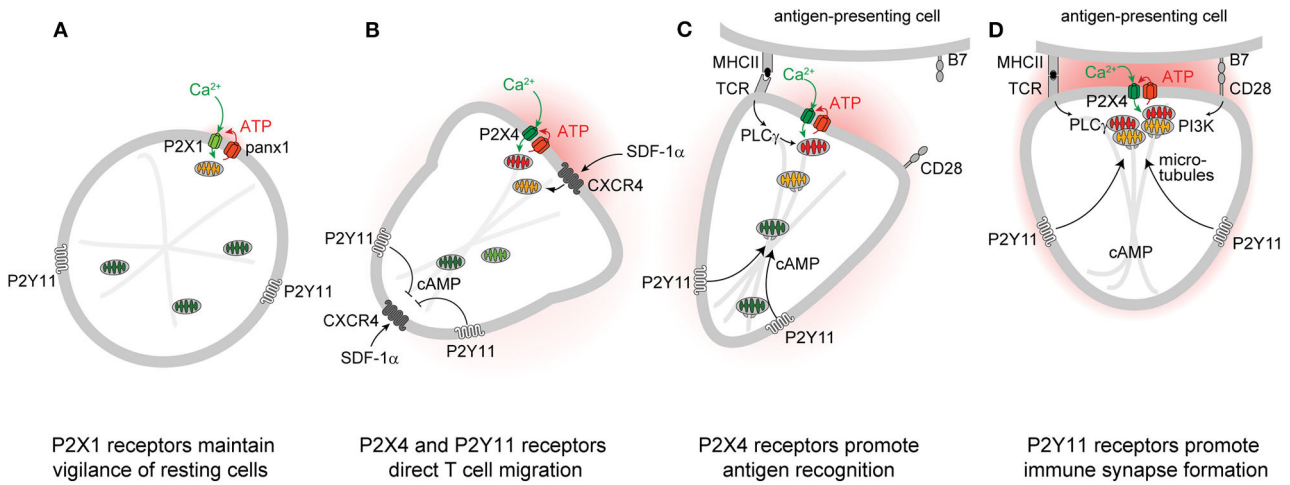

FIGURE 1 | P2 receptors and mitochondria regulate key T cell functions. Autocrine feedback through P2X1 receptors and low-level mitochondrial metabolism maintain a state of vigilance that quiescent T cells need for immune surveillance **(A)**. Chemokine receptors such as CXCR4 trigger mitochondrial metabolism that stimulates P2X4 and P2Y11 receptor-mediated excitatory and inhibitory Ca^{2+} and cAMP signaling pathways that direct cell movement at the front and back of migrating T cells **(B)**. P2X4 receptor accumulation at the immune synapse enhances T cell receptor (TCR) signaling and promotes antigen recognition and the engagement of T cells with antigen-presenting cells **(C)**. P2Y11 receptor recruitment to the uropod of polarized cells induces cAMP/PKA signaling that helps direct the trafficking of mitochondria to the immune synapse **(D)**.

Similar mitochondrial/purinergic feedback loops also orchestrate the migration of other immune cell subtypes (54–58). Like T cells, neutrophils depend on excitatory purinergic receptors, panx1 channels, and mitochondria to coordinate different aspects of their migration in chemotactic gradient fields (6, 55). However, neutrophils differ from T cells in that P2Y2 receptors rather than P2X4 receptors amplify the chemotactic signals that direct cell migration at their leading edge (6, 54). Microglia, macrophages, and dendritic cells also depend on autocrine feedback mechanisms and specific purinergic receptors to regulate cell migration (56–58). Recent studies have shown that inhibition of the mitochondrial electron transport chain impairs the motility of neutrophils in zebrafish (59). Thus, mitochondrial metabolism and purinergic signaling seem to be preserved features that regulate immune cell migration in humans and other vertebrates.

P2Y11 RECEPTORS CONTRIBUTE TO T CELL MIGRATION BY RESTRAINING MITOCHONDRIAL METABOLISM

According to the local excitation—global inhibition (LEGI) model of chemotaxis, excitatory mechanisms at the front elicit cell protrusion, while inhibitory mechanisms at the back promote the retraction of the cell body during cell migration (60–62). In neutrophils, P2Y2 receptors provide the excitatory signal at the front, while A2a adenosine receptors generate the inhibitory cAMP/PKA signal that causes cell retraction at the back of cells (63). In T cells, P2X4 and P2Y11 receptors fulfill similar roles in the regulation of cell migration (12, 64). Like the A2a receptors of neutrophils, the P2Y11 receptors of T cells can couple to $G\alpha_s$ proteins that trigger cAMP/PKA signaling pathways (65). P2Y11 receptors bind their natural ligand, ATP, with a reported EC_{50} value of 2.5 to 63 μM, which is similar to the affinity of P2X4 receptors (41). Therefore, the pericellular ATP that surrounds stimulated T cells can trigger both P2X4 receptor-mediated Ca^{2+} influx and P2Y11 receptor-mediated cAMP/PKA signaling that restrains excitatory signaling and transduction pathways downstream of $G\alpha_{i/o}$-coupled GPCRs like CXCR4 (66, 67). We found that P2Y11 receptors redistribute to the back of polarized T cells where they induce cAMP/PKA signaling events that stabilize cell polarization by locally restricting cell stimulation by CXCR4 chemokine receptors at the back (**Figure 1B**) (64). Thus, P2X4 and P2Y11 receptors synergize to regulate mitochondrial metabolism and provide T cells with the local excitation and global inhibition cues that organize pseudopod protrusion and uropod retraction during T cell migration in a LEGI-type fashion.

P2Y11 AND P2X4 RECEPTORS ORCHESTRATE THE ACCUMULATION AND ACTIVATION OF MITOCHONDRIA AT THE IMMUNE SYNAPSE OF T CELLS

T cells must interact with APCs in order to mount immune responses. These interactions occur via organized structures referred to as immune synapses (IS) that consist of microclusters containing T cell receptors (TCR), CD3, CD28 co-receptors, LAT, SLP76, LFA-1, microtubules, and other cytoskeletal components (68). The formation of a stable IS between a T cell and an APC enables sustained TCR signaling that culminates in cytokine production and T cell proliferation (69). Efficient T cell activation also depends on sustained Ca^{2+} influx from the extracellular space (70). Just minutes after TCR stimulation, P2X4 receptors, panx1 channels, and mitochondria accumulate at

the IS where mitochondria generate the ATP that panx1 channels release into the synaptic cleft to stimulate P2X4 receptor-mediated Ca^{2+} influx (36, 71, 72). P2X4 receptors deliver the Ca^{2+} that mitochondria need to synthesize ATP via oxidative phosphorylation (73). However, mitochondria also act as Ca^{2+} sinks that fine-tune cytosolic Ca^{2+} levels for efficient T cell activation (74). Thus, mitochondria, panx1, and P2X4 receptors represent a powerful feedforward signaling system that triggers downstream pathways that involve mitogen-activated protein kinases (MAPKs) and nuclear factors of activated T cells (NFAT) and induce IL-2 transcription and T cell proliferation (10, 11, 36).

Successful T cell activation depends on the accumulation of mitochondria at the IS (71, 72, 75). However, the mechanisms that orchestrate mitochondrial trafficking to the IS are not clear (76). In neurons, kinesin and dynein motors accomplish anterograde and retrograde trafficking of mitochondria along microtubules (77). In T cells, dynein facilitates mitochondrial transport to contact sites that T cells form with endothelial cells during their transmigration across blood vessel walls (78). Dynamin-related protein 1 (DRP1) is a mitochondrial fission factor that helps direct mitochondria to the uropod of migrating T cells and to the IS during APC engagement (45, 75). In neurons, cAMP promotes directional movement of mitochondria along the microtubule network (79–82), while local cytosolic Ca^{2+} hotspots act as mitochondrial stop signals (83). Our recent work has shown that P2Y11 receptors promote trafficking of mitochondria to the IS of T cells (84). Thus, P2Y11 and P2X4 receptors jointly recruit and activate mitochondria at the IS in order to sustain T cell activation. However, further studies are needed to reveal the detailed mechanisms by which these purinergic receptors, motor proteins, and the microtubule network regulate the complex process that energizes the IS in T cells (**Figures 1C,D**).

Several lines of evidence indicate that purinergic signaling has important physiological implications for *in vivo* T cell functions. Consistent with the critical roles of P2X receptors in T cells, genetic variants of P2X4 and P2X7 receptors were found to contribute to multiple sclerosis, a T cell-mediated inflammatory autoimmune disease (85). Furthermore, CD4 T cell infiltration into the spinal cord of mice subjected to experimental autoimmune encephalomyelitis is attenuated in *Panx1* knockout mice (53). The significance of P2Y11 receptors as regulators of human immune responses is supported by recent findings that single nucleotide polymorphisms (SNPs) in the P2Y11 receptor gene are associated with inflammatory disorders that increase the risk of acute myocardial infarction and predispose patients to narcolepsy and reduced T cell viability (86, 87).

SYSTEMIC ATP ACCUMULATION IMPAIRS IMMUNE CELL FUNCTIONS BY INTERFERING WITH THEIR AUTOCRINE PURINERGIC SIGNALING MECHANISMS

T cells travel to lymphoid organs and other host tissues where they interact with APCs in order to elicit effector functions needed for host defense. As outlined above, T cell functions depend on intricate autocrine signaling mechanisms to execute their roles in host defense. However, these autocrine signaling mechanisms are susceptible to paracrine interference by exogenous ATP that accumulates in response to cell damage, tissue injury, or inflammation. Systemic ATP levels also increase in sepsis and in the tumor microenvironment, which impairs T cell migration, cytokine production, and T cell proliferation (**Figure 2**) (16, 88, 91–93). Global and disproportionate stimulation of P2X1, P2X4, and P2Y11 receptors across the cell surface disrupts the spatiotemporal sequence of the autocrine purinergic signaling events that regulate T cells and host immune functions (64, 94).

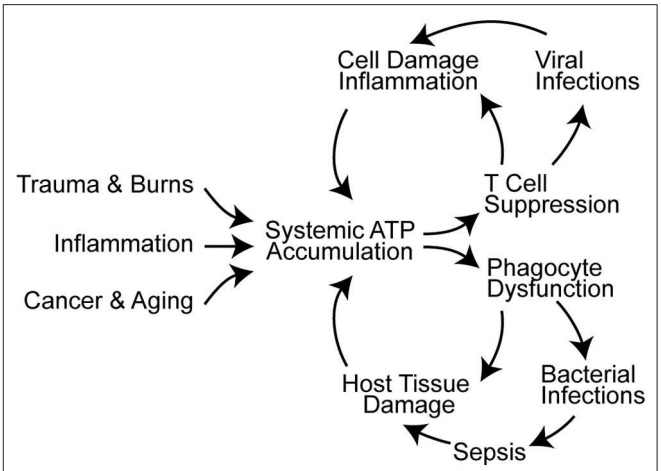

FIGURE 2 | Systemic ATP accumulation impairs the autocrine purinergic signaling mechanisms that regulate immune functions. Trauma, burns, inflammation, cancer, and aging are associated with systemic ATP accumulation that promotes immune cell dysfunction (16, 88–90). This results in infections, sepsis, and additional cell damage that exacerbates systemic ATP levels and propagates immune dysfunction.

Besides P2X1 and P2X4 receptors, T cells also express the P2X7 receptor subtype. P2X7 receptors are comparatively insensitive to ATP with an EC_{50} value of ~780 μM (41). Interestingly, P2X7 receptors remain uniformly distributed across the cell surface of T cells even during IS formation with APCs (36). This suggests that P2X7 receptors may act primarily as mediators of paracrine rather than autocrine ATP signaling. P2X7 receptor stimulation by external ATP can alter the composition of T cell subpopulations by promoting the Th1/Th17 differentiation of CD4 T cells, the conversion of immunosuppressive regulatory T cells (T_{regs}) into proinflammatory Th17 cells, and the formation of long-lived CD8 memory T cell subsets (37, 95). However, P2X7 receptors may also contribute to the onset of autoimmune diseases such as type 1 diabetes, namely by enhancing the activation of autoreactive CD8 effector T cells (96). P2X7 receptors differ from other purinergic receptors in that they form large and unselective macropores in response to millimolar ATP concentrations, which ultimately results in cell death (33). Physiologically, this enables P2X7 receptors to control T follicular helper (Tfh) cell numbers in Peyer's patches of the small intestine and to modulate the

production of IgA that shapes the gut microbiota composition (97). P2X7 receptor stimulation also limits the expansion of autoreactivity-promoting Tfh cells, whereas Tfh cells that respond to cognate antigens are protected from P2X7 receptor-mediated cell death (97–99). On the other hand, P2X7 receptor-mediated cell death may also contribute to the suppression of T cell immunity in the presence of pathologically elevated systemic ATP levels.

Excessive ATP in the systemic environment of neutrophils has similarly disruptive implications on cell functions. Overstimulation of excitatory P2Y2 receptors disrupts neutrophil chemotaxis and bacterial clearance. At the same time, excessive P2Y2 receptor stimulation by systemic ATP aggravates inflammatory neutrophil responses such as oxidative burst and degranulation, which culminate a in neutrophil-mediated collateral host tissue damage (**Figure 2**) (100–102). Systemic ATP may have a similar impact on other immune cells including macrophages that depend on P2X4 and P2X7 receptors for bacterial clearance in polymicrobial sepsis (103, 104). Targeting extracellular ATP could be a promising approach to overcome systemic inflammation and immunosuppression in critical care and cancer patients. The therapeutic potential of this approach is supported by observations that treatment with apyrase and other enzymes that hydrolyze extracellular ATP can indeed improve outcome in mouse models of inflammation and sepsis (89, 102, 105).

CONCLUDING REMARKS

Breakdown of increased systemic ATP levels can elevate extracellular adenosine concentrations. Adenosine exerts mostly anti-inflammatory effects through A2a and A2b receptors. While adenosine can protect tissues from inflammatory damage, excessive adenosine signaling contributes to immunosuppression in cancer and sepsis (106). The suppressive effect of A2a receptor stimulation on various T cell functions has been studied in great detail in mice (107). CD39 and CD73 are dominant enzymes responsible for the conversion of ATP to adenosine. Both ectonucleotidases are highly expressed by murine T_{regs} that suppress T cell functions by generating adenosine and stimulating A2a receptors (27, 32). In contrast to mice, CD39 expression on human CD4 T cells is largely restricted to memory T_{regs} (108), and T cell inhibition by adenosine receptor-dependent pathways seems to be less important in humans than in mice (109). Interestingly, mice and other rodents do not possess P2Y11 receptors (110). Thus, mouse models cannot fully reflect human disease processes. It seems likely that A2 adenosine receptors in mice fulfill the roles of human P2Y11 receptors in the regulation of T cell functions. These species-specific differences must be considered during the development of treatments for inflammatory, infectious, and other T cell-centered diseases such as cancer.

AUTHOR CONTRIBUTIONS

CL and WJ prepared the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Targeting Hypoxia-A2A Adenosinergic Immunosuppression of Antitumor T Cells During Cancer Immunotherapy

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The blockade of immunological negative regulators offered a novel therapeutic approach that revolutionized the immunotherapy of cancer. Still, a significant portion of patients fail to respond to anti-PD-1/PD-L1 and/or anti-CTLA-4 therapy or experience significant adverse effects. We propose that one of the major reasons that many patients do not respond to this form of therapy is due to the powerful physiological suppression mediated by hypoxia-adenosinergic signaling. Indeed, both inflamed and cancerous tissues are hypoxic and rich in extracellular adenosine, in part due to stabilization of the transcription factor hypoxia-inducible factor 1 alpha (HIF-1α). Adenosine signals through adenosine A2A receptors (A2AR) to suppress anti-tumor and anti-pathogen immune responses. Several classes of anti-hypoxia-A2AR therapeutics have been offered to refractory cancer patients, with A2AR blockers, inhibitors of adenosine-generating enzymes such as CD39 and CD73, and hypoxia-targeting drugs now reaching the clinical stage. Clinical results have confirmed preclinical observations that blockade of the hypoxia-adenosine-A2AR axis synergizes with inhibitors of immune checkpoints to induce tumor rejection. Thus, A2AR blockers provide a new hope for the majority of patients who are nonresponsive to current immunotherapeutic approaches including checkpoint blockade. Here, we discuss the discoveries that firmly implicate the A2AR as a critical and non-redundant biochemical negative regulator of the immune response and highlight the importance of targeting the hypoxia-adenosine-A2AR axis to manipulate anti-pathogen and anti-tumor immune responses.

Keywords: adenosine, hypoxia, cancer immunotherapies, T cell, HIF−1α, immune checkpoint, immunology

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OVERVIEW OF THE HYPOXIA-ADENOSINE-A2AR AXIS

While hypoxia-dependent generation of extracellular adenosine and subsequent immunosuppressive signaling through adenosine A2A receptors (A2AR) is deleterious in the tumor microenvironment (TME), this mechanism normally has an important tissue-protective function. The suppression of tumor-reactive T cells by hypoxia-adenosine-A2AR signaling in the TME is a commandeering of this evolutionarily conserved, non-redundant feedback mechanism to govern inflammation (1–3). Sitkovsky and colleagues were the first to confirm *in vivo* that this may explain the paradoxical peaceful coexistence of tumors and antitumor T cells in tumors (4, 5).

These studies demonstrated that A2AR signaling inhibited important effector functions of T cells, such as secretion of pro-inflammatory cytokines (e.g., IFNγ) (6). However, the anti-inflammatory effects of the hypoxia-adenosine-A2AR axis have been confirmed and extended to include suppression of T cell proliferation, cytotoxicity, and induction of anti-inflammatory cytokine secretion (e.g., IL-10) (7–9).

The hypoxia-adenosine-A2AR axis of immunosuppression begins with hypoxia and the stabilization of hypoxia-inducible factor-1alpha (HIF-1α), which increases extracellular adenosine in part by upregulating adenosine-generating enzymes. Subsequent signaling through the Gs-coupled/cAMP-elevating A2ARs induces protein kinase A (PKA)-mediated inhibition of T-cell receptor signaling and immunosuppressive transcriptional changes (10). This includes the inhibition of pro-inflammatory cytokine secretion and an increase in the levels of anti-inflammatory cytokines that contain a cAMP response element (CRE) consensus sequence in their respective promoter regions. While adenosine can also activate cAMP-elevating adenosine A2B receptors (A2BRs), our research has focused on A2AR adenosinergic immunosuppression due to a higher affinity for adenosine and higher expression on T cells (11–13). Importantly, A2AR expression seems to be the limiting factor in adenosine-mediated cAMP generation in T cells since there is no receptor reserve of A2AR (14). T cells can also possess a memory of A2AR signaling, allowing the effects of adenosine to persist long after exposure (15).

Adenosine also exerts immunosuppressive effects through A2BR, particularly on innate immune cells. Groundwork for this hypothesis can be found in studies demonstrating that adenosinergic immunosuppression of IL-12 and TNFα by macrophages is at least partially A2AR-independent (16). For example, in lipopolysaccharide-stimulated macrophages, A2BR activation increases anti-inflammatory IL-10 production by attenuating translational arrest of IL-10 mRNA (17). Conversely, A2BR signaling may enhance activation of alternative/Th2 cytokine-activated macrophages, which manifest several anti-inflammatory functions (18). In group 2 innate lymphoid cells (IL2C), adenosine has been demonstrated to decrease IL-5 and IL-13 production through A2BR, but increase IL-5 production through A2AR. Activation of both A2AR and A2BR in IL2C results in a net decrease in IL-5 production, indicating the importance of A2BR on this cell type (19). Interestingly, HIF-1α-dependent expression of A2BR has also been shown to induce the enrichment of breast cancer stem cells (20). Additional studies of preclinical models of acute lung injury have also demonstrated that an increase in HIF-1α levels in pulmonary epithelia subjected to cyclic mechanical stretch resulted in an increase in A2BR expression (21). A2BR-mediated immunosuppression of a variety of immune cells, including dendritic cells, has led to the development of dual A2AR/A2BR antagonists which may prevent adenosinergic immunosuppression of both innate and adaptive immune cells (22).

The main metabolic precursor to adenosine is ATP. Under homeostatic conditions, ATP is magnitudes higher intracellularly than in the extracellular space (23, 24). However, in inflamed and cancerous tissues, apoptotic and necrotic cells release ATP into the extracellular compartment, disrupting this gradient (25). Excess ATP is then degraded into adenosine by CD39/CD73 (26–29), CD38/CD203a (30–33) and other phosphatases in certain tissues (28). While the primary mechanism is thought to be mediated by CD39 and CD73 (34), alternative adenosine-generating pathways, such as CD38, are an important contributor to adenosine levels in the TME and inhibit antitumor T cells via A2AR. Indeed, recent studies have demonstrated that PD-1 blockade can increase CD38 expression, leading to resistance to αPD-1 therapy (35).

Consistent with findings regarding adenosine-A2AR immunosuppression, multiple studies from different teams have confirmed the tissue-protecting roles of CD39 and CD73. CD39, which converts ATP to AMP, also serves an anticoagulant function in vasculature (36). Indeed, CD39 has been demonstrated to attenuate both renal ischemia and acute lung injury (37, 38). CD73, which converts AMP to adenosine, has also been shown to have a role in the mediation of cell adhesion to endothelium (39). Moreover, some tumorigenic functions of CD73 have been shown to be independent of its enzymatic function, such as induction of angiogenesis (40). Interestingly, recent studies have also shown that A2AR signaling can promote angiogenesis, suggesting a role for the HIF-1α-CD73-adenosine-A2AR axis in tumor-associated lymphangiogenesis and metastasis (41).

The upstream portion of the hypoxia-adenosine-A2AR axis is mediated by hypoxia/HIF-1α. HIF-1α upregulates genes containing an hypoxia response element (HRE) consensus sequence that mediates cell survival in hypoxic conditions. The immunosuppressive role of HIF-1α was first implicated in studies of HIF-1α$^{-/-}$ Rag-2$^{-/-}$ mice with HIF-1α deletion in T cells and B cells. These experiments demonstrated that HIF-1α regulates lymphocyte development and prevents autoimmunity (42). Subsequent studies of mice with T cell-specific HIF-1α deletion confirmed an immunosuppressive role for HIF-1α. These mice exhibited an enhanced antibacterial response due to the lack of HIF-1α-mediated inhibition of T cells (43). Studies that prevent HIF-1α stabilization using supplemental oxygenation have also provided direct mechanistic evidence for HIF-1α-mediated upregulation of the hypoxia-adenosine-A2AR axis (44). It must be emphasized that upregulation of CRE-containing genes and HRE-containing genes may not be mutually exclusive. The gene encoding the characteristic regulatory T-cell transcription factor FoxP3, which upregulates HIF-1α, is induced by CRE activation (45, 46). Thus, it is suggested that crosstalk exists between CRE and HRE pathways and they may synergize to strengthen immunosuppression (47, 48). Physiologically, this is supported by the infectious tolerance mediated by regulatory T cells in inflamed and cancerous tissues (49–51).

PHARMACOLOGICAL TARGETS IN THE HYPOXIA-ADENOSINE-A2AR AXIS FOR CANCER IMMUNOTHERAPY

A2ARs

Inquiry into the immunosuppressive functions of adenosine was catalyzed by the established importance of cAMP as an immunosuppressive agent (52). cAMP has been demonstrated to inhibit many effector T cell functions via PKA activation (53–59). Landmark studies by Sitkovsky provided the first genetic and pharmacological evidence that the cAMP-elevating A2AR has a critical and non-redundant immunosuppressive role in tissue protection during excessive inflammation (6). These studies also offered insights into why antitumor T cells often fail to mount an effective response against cancerous tissue. Indeed, tumors are rich in extracellular adenosine, in large part due to poor, irregular vasculature resulting in local hypoxia (60–62). The tumor-protecting role of A2AR was conclusively established using mice with A2AR gene deletion (5). This study also complemented genetic evidence with pharmacological data, demonstrating that A2AR antagonism or silencing by siRNA enhanced the efficacy of adoptive cell transfer (ACT) (5). This was supported by follow-up studies demonstrating that A2AR antagonism during ACT or adoptive transfer of A2AR-deficient T cells were effective approaches for enhancing the efficacy of ACT in mice (63). The therapeutic benefit of A2AR antagonism was shown to be due in part by increased IFNγ secretion by tumor-infiltrating adoptively transferred T cells (63). Importantly, this study also demonstrated that A2AR antagonism improved anti-tumor immunity independent of the anatomical location of the tumor and provided long-term tumor-specific memory (63). Taken together, these studies provided proof of principal for the use of A2AR antagonists during cancer immunotherapies, particularly ACT.

The progress in methods of ACT and the studies reviewed above offered justification to test whether CAR-T cells might also be susceptible to hypoxia-adenosinergic immunosuppression. It has been hypothesized that A2AR blockade may improve efficacy of CAR-T therapies against cancers. This may prove essential for CAR-T that target solid tumors, which are known to be hypoxic and extracellular adenosine-rich. Indeed, early evidence was provided by Albelda's group demonstrating that genetic engineering to prevent PKA trafficking to the CAR-T cell membrane enhanced antitumor function *in vivo* and conferred resistance to adenosinergic immunosuppression *in vitro* (64). Critical studies by Darcy's Team demonstrated that both pharmacological and genetic inhibition of A2AR enhanced CAR-T efficacy in two distinct murine models of syngeneic breast cancer. Of clinical relevance, addition of αPD-1 to the CAR-T/A2AR blockade protocol further enhanced CAR-T efficacy, as indicated by increased IFNγ production by CAR-T (65). These findings confirm and extend the observations that A2AR antagonism enhances production of IFNγ by polyclonal adoptively transferred T cells in the TME to improve tumor regression (63).

Pioneering studies by Powell's Team established that A2AR agonism can upregulate negative regulators of the immune response such as LAG-3 (8). Subsequent studies using the A2AR antagonist CPI-444 have also provided strong justification for A2AR blockade during cancer immunotherapies. These studies confirmed and extended observations of improved antitumor efficacy of ACT in combination with A2AR blockade. Additional mechanistic evidence justifying A2AR blockade was provided by demonstrations that A2AR blockade reduced PD-1 and LAG-3 expression on effector and regulatory T cells, as well as reduced expression of these immune checkpoint molecules in tumor-draining lymph nodes (66). Taken together, these findings indicate that A2AR blockade can prevent inhibition of already active antitumor T cells, and also prevent inhibition during initial activation (66). Consistent with this finding, it has also been demonstrated that A2AR deletion increases terminally mature natural killer cells in the TME, implicating adenosine as a negative regulator of innate immune cell maturation as well (67). Important studies by Miller and Willingham in multiple preclinical cancer models confirmed that combining A2AR antagonism with checkpoint blockade improved tumor regression, strengthening mechanistic evidence to justify clinical testing of this approach (68). *In vitro* assays also demonstrated that CPI-444 prevented adenosinergic inhibition of IL-2 and IFNγ production by T cells (68). Through analysis of gene expression, these studies were also able to identify a Th1 expression signature that was associated with positive responses to dual blockade of A2AR/PD-L1 (68).

These preclinical studies have led to the clinical testing of A2AR antagonists as a cancer therapy and have yielded promising results. Against renal cell cancer, A2AR antagonism using CPI-444 induced durable responses both as a monotherapy and when combined with the PD-L1 inhibitor atezolizumab. Patients experiencing positive responses included individuals who had previously shown resistance to αPD-L1 therapy. Consistent with preclinical data, alleviation of adenosinergic immunosuppression resulted in higher cytotoxic T cell tumor infiltration. This study also elucidated a gene-expression signature that was associated with positive response (69). In another clinical study, the A2AR antagonist NIR178 administered both as a monotherapy and in combination with the PD-1 inhibitor spartalizumab to 24 non-small lung cancer patients resulted in stable disease in fifteen patients in addition to one partial response and one complete response (70). Furthermore, the A2AR antagonist AZD4635 used as a monotherapy and in combination with the PD-L1 inhibitor durvalumab induced strong responses in three of eight metastatic castration-resistant prostate cancer patients (71). These tumors may be naturally adenosine-rich due to prostatic acid phosphatase activity and therefore a good candidate for A2AR blockade (71).

CD39/CD73

It has been established that CD39/CD73 also have a major role in facilitating immune escape by tumors. Indeed, Robson's Team established the field of CD39 and were the first to demonstrate that CD39 deletion alleviated tumor burden in a preclinical

model of hepatic metastatic cancer (72). Parallel studies by Smyth's Team also demonstrated that administration of a CD73 monoclonal antibody (mAb) decreased tumor burden in two distinct murine tumor models. This approach also suggested that not only did CD73 inhibit antitumor leukocytes via adenosine generation, but affected tumor metastasis as well (73). Moreover, Stagg's Team demonstrated that CD73 overexpression in human triple-negative breast cancer correlated with poor prognosis and resistance to chemotherapy in a preclinical model of breast cancer (74). Important studies by Smyth's Team also demonstrated improved anti-tumor efficacy using an A2AR antagonist in combination with a CD73 inhibitor to alleviate tumor burden (75). These findings also highlight the importance of targeting multiple components of the hypoxia-adenosine-A2AR axis. Indeed, small molecule inhibitors or monoclonal antibodies against CD39 and CD73 are emerging as potent anti-cancer therapies (49, 74, 76–82). Furthermore, αCD73 therapy has been demonstrated to improve the therapeutic benefit of αPD-1/αCTLA-4 therapy in multiple preclinical cancer models (80).

Several mAb CD73 inhibitors have exhibited strong antitumor efficacy in clinical trials with findings consistent with preclinical data. In 66 pancreatic or colorectal cancer patients, the αCD73 mAb MEDI9447 as monotherapy and in combination with durvalumab decreased CD73 expression on peripheral T cells. In addition, MEDI9447 decreased CD73 expression in five out of nine tumors, which correlated with increased cytotoxic T cell infiltration (83). The αCD73 mAb BMS986179 as a monotherapy and in combination with the PD-1 inhibitor nivolumab also induced partial responses or stable disease in 17 of 59 patients with various malignancies (84).

HIF-1α

Given the hypoxia-HIF-1α-mediated upregulation of adenosine-generating enzymes, Sitkovsky's Team established in decades-long studies that hypoxia-HIF-1α inhibits T cells (10). It was then hypothesized and confirmed that the reversal of hypoxia could prevent the inhibition of antitumor T cells by hypoxia-adenosine-A2AR-mediated immunosuppression. Indeed, preclinical studies demonstrated that supplemental oxygen (60% O_2) decreased levels of hypoxia, HIF-1α, and extracellular adenosine in the TME (44). This was supported by data demonstrating oxygenation-mediated reduction in CD39, CD73, A2AR, A2BR, and COX-2 expression (44). Importantly, supplemental oxygen was also shown to upregulate MHC class I expression by tumor cells, allowing for increased recognition and subsequent elimination by antitumor T cells (44). Parallel studies demonstrated the immunological effects of supplemental oxygen by showing that oxygenation converts an immunosuppressive TME to an immunopermissive TME. This resulted in an increase in many pro-inflammatory cytokines as well as recruitment of endogenous and adoptively transferred antitumor T cells into the TME. This was also accompanied by a reduction in many anti-inflammatory molecules such as TGFβ, CTLA-4, and FoxP3, as well as an overall reduction in regulatory T cells in the TME (85). This resulted in significant tumor regression and long-term survival in preclinical tumor models. Importantly, these studies also established that the reversal of hypoxia improved the efficacy of immune checkpoint blockade with αCTLA-4/αPD-1 (85).

HIF-1α can also be pharmacologically targeted using small molecule drugs such as digoxin, acriflavine, and ganetespib. Indeed, these drugs have shown efficacy in preclinical tumor models (86–88). While the immunosuppressive effects of HIF-1α have been shown to be mediated in part by hypoxia-adenosinergic signaling, HIF-1α also has other non-adenosinergic immunosuppressive effects (89). Additionally, immunosuppression via adenosine-A2AR signaling may not be completely reversed by only targeting hypoxia/HIF-1α. Therefore, an ideal approach for completely abrogating the immunosuppressive effects of the hypoxia-adenosine-A2AR axis might be the co-administration of both anti-hypoxia-HIF-1α therapies and A2AR antagonists during cancer immunotherapy (90).

CONCLUSION

The hypoxia-adenosine-A2AR axis is a potent inhibitor of antitumor T cells. This pathway presents multiple pharmacological targets. Of particular importance and translational value are A2ARs, CD39/CD73, and HIF-1α. Inhibition of this pathway has been shown to enhance the efficacy of current cancer immunotherapy approaches, including αCTLA-4/αPD-1. Multiple studies have reported synergism between checkpoint inhibitors and several classes of anti-hypoxia-adenosine-A2AR therapeutics. Our preclinical studies provided the rationale and justification for combining A2AR blockade and supplemental oxygen/oxygenation agents during cancer immunotherapies. We postulate that this approach will maximize the efficacy of the antitumor immune response in clinical studies.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Adenosine at the Interphase of Hypoxia and Inflammation in Lung Injury

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Hypoxia and inflammation often coincide in pathogenic conditions such as acute respiratory distress syndrome (ARDS) and chronic lung diseases, which are significant contributors to morbidity and mortality for the general population. For example, the recent global outbreak of Coronavirus disease 2019 (COVID-19) has placed viral infection-induced ARDS under the spotlight. Moreover, chronic lung disease ranks the third leading cause of death in the United States. Hypoxia signaling plays a diverse role in both acute and chronic lung inflammation, which could partially be explained by the divergent function of downstream target pathways such as adenosine signaling. Particularly, hypoxia signaling activates adenosine signaling to inhibit the inflammatory response in ARDS, while in chronic lung diseases, it promotes inflammation and tissue injury. In this review, we discuss the role of adenosine at the interphase of hypoxia and inflammation in ARDS and chronic lung diseases, as well as the current strategy for therapeutic targeting of the adenosine signaling pathway.

Keywords: adenosine, inflammation, hypoxia, hypoxia-inducible factor, acute lung injury, chronic lung injury

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is common in critically ill patients, characterized by respiratory failure, pulmonary edema independent of left heart failure, as well as high morbidity and mortality (1). The mortality rate was 30–40% in the most recent studies despite the latest improvement in clinical management (2). Pathological characters of ARDS in the acute "exudative" phase (~7 days) include alveolar epithelial and endothelial injury, resulting in interstitial and alveolar edema, hyaline membrane formation, and alveolar hemorrhage, as well as the accumulation of immune cells (1, 3). The main causes for ARDS include pneumonia, aspiration of gastric contents, severe trauma as well as sepsis (1, 4, 5). The recent global outbreak of Coronavirus disease 2019 (COVID-19) has placed viral infection-induced ARDS under the spotlight. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and has resulted in a worldwide pandemic rapidly because of its high transmissibility and pathogenicity (6). ARDS is one of the most common organ dysfunctions for severe COVID-19, which accounts for the cause of death in 70% of fatal cases (7, 8). There are several emerging viruses in the past 20 years, which can induce ARDS-related mortality, such as influenza H1N1 2009,

influenza H5N1 and H7N9 viruses, the severe acute respiratory syndrome coronavirus (SARS), and Middle East respiratory syndrome coronavirus (MERS) (9). It is reported that about 30–40% of the hospitalized patients infected with influenza virus progress to pneumonia, and influenza A shows a higher predisposition to ARDS in adults (10). Compared to SARS (10%) and MERS (35%), COVID-19 shows lower mortality rates of approximately 5.2%, but higher infectiousness (9, 11). As of September 6^{th,} 2020, the pandemic of COVID-19 had affected over 26 million individuals around the world and caused more than 800,000 deaths worldwide. Therefore, the search for effective therapeutic approaches for the preventing and treatment of COVID-19 associated ARDS has become an urgency. Currently, although there are certain improvements in the management of ARDS, the treatment for ARDS is in urgent need. Therefore, the fundamental pathogenesis and effective treatments for ARDS are still under intensive investigation.

Persistent pulmonary inflammation and tissue remodeling result in the gradual decline in pulmonary function in patients suffering from chronic lung diseases including chronic obstructive pulmonary disease (COPD), asthma, and idiopathic pulmonary fibrosis (IPF) (3, 12–16). Chronic lung disease ranks the third leading cause of death in the United States. The risk factors of chronic lung diseases included genes, environmental factors, and aging (3, 12, 13, 17). However, one of the common characteristics among these diseases is dysregulated recruitment or activation of immune cells, such as neutrophils, macrophages, dendritic cells, and other effector cells, such as fibroblasts, myofibroblasts, and airway epithelial cells (AECs), which accelerates pulmonary remodeling and inflammatory response (3, 4). The therapeutic approaches for chronic lung diseases focus on providing symptomatic relief, but pharmacologic compounds are still lacking to reverse the profound tissue remodeling and restore lung function in these patients.

Adenosine was first isolated from the heart muscle and identified as an "adenine compound" that could change cardiac rhythm when injected in guinea pigs in 1927 (18). Besides its function in cardiac rhythm, adenosine also modulates inflammatory responses during hypoxic conditions (19–21). In this review, we will discuss the interaction between hypoxia and adenosine signaling, including adenosine, adenosine receptors, and adenosine metabolism, in acute lung inflammation and chronic lung diseases. We will also focus on the currently available approaches for therapeutic targeting of the hypoxia-adenosine axis in these disease conditions.

BIOLOGY OF ADENOSINE

Extracellular Adenosine Generation

Adenosine, along with ATP and ADP, is considered the main purinergic signaling molecules (**Figure 1**). The release of ATP from intracellular to the extracellular environment contributes to the formation of adenosine especially when the tissue is in inflammatory, ischemic, and hypoxic conditions (23, 24). ATP/ADP in the extracellular space can be converted to adenosine monophosphate (AMP) by ectonucleoside triphosphate diphosphohydrolase-1 (CD39) (25, 26). Then AMP is further converted by ecto-5'-nucleotidase (CD73) to extracellular adenosine (25, 26). Mice with CD39 or CD73 deficiency are viable, which indicates that nucleotide phosphohydrolysis regulated by ectoenzymes is not vital in regular physiologic conditions (27). However, ectonucleotidases still have a crucial role in disease conditions. For example, the upregulation of adenosine generation and CD39 and CD73 expression is one of the protective mechanisms to reduce apoptosis, and alleviate inflammation in kidney ischemia/reperfusion (I/R) injury models (28). The deletion of CD39 in mice leads to increased level of ATP/ADP, and reduced adenosine levels, along with elevated risk of dysregulated inflammation and tissue injury (29, 30). Similarly, genetic deletion of CD73 results in higher mortality and delayed acute lung injury resolution when compared with WT mice because of the dampened generation of adenosine in regulatory T cells (Tregs) (31). Therefore, the conversion of ATP/ADP to adenosine is considered beneficial in many ischemic and inflammatory disorders.

Adenosine Receptors and Signaling

Adenosine receptors, which include four distinct G-protein coupled seven membrane-spanning cell surface receptors: the adenosine A_1 receptor (A1AR), the adenosine A_{2A} receptor (A2AAR), the adenosine A_{2B} receptor (A2BAR), and the adenosine A_3 receptor (A3AR), are crucial for adenosine mediated responses (3, 19, 21, 27). Both A2AAR and A2BAR are linked to Gs protein involving activation of adenylate cyclase, to stimulate cAMP production followed by PKA activation (32–35). A1AR and A3AR, on the other hand, bear a distinct signal transduction pathway. For example, A1AR activation inhibits cAMP accumulations in Chinese hamster ovary cells (36). The coupling of A1AR to the Gi/o protein pathway attenuates cAMP signal transduction in hepatic stellate cells (33). Furthermore, A3AR has been indicated to attenuate adenosine-induced increase of cAMP in rat vascular smooth muscle cells *in vitro* (37) and A3AR knockout mice show an increased level of cAMP in the cardiovascular system (38). Functionally, Dr. Michail Sitkovsky's laboratory identified that A2AAR is crucial for limiting inflammatory responses as mice with A2AAR deficiency showed profound tissue damage in inflammation and endotoxin-induced septic shock (21). The expression of adenosine receptor subtypes is different in various cell types. For example, neutrophils and lymphocytes have higher expression levels of A2AAR, while vascular endothelial cells have higher levels of A2BAR (39–41). It has been elucidated that adenosine receptors have important functions in pathologic conditions. For instance, adenosine has a selective role in reducing the heart rate *via* A1AR, which would be a potential therapeutic method for superventricular tachycardia in mice (42). Adenosine signaling *via* A2AAR or A2BAR has a beneficial effect *via* shifting proinflammatory immune response to anti-inflammatory immune response as well as promoting barrier protection in different animal models (43–48). A3AR is related to the aqueous humor production in the eye in a preclinical study (49), and its agonist showed efficacy in treating dry eye syndrome in a clinical study (50).

Intracellular Adenosine Metabolism

The termination of adenosine signaling is mediated by the transportation of adenosine from the extracellular to the intracellular space (**Figure 1**) (27, 51). ENTs and concentrative nucleoside transporters (CNTs) are nucleoside transporters found on various cell types (52, 53). According to the concentration gradient, adenosine moves freely across these channels because of its diffusion-limited character (53). Adenosine signaling can be diminished by the transportation of adenosine into the cell and then metabolized to inosine *via* adenosine deaminase (ADA) (54). Additionally, adenosine kinase can convert adenosine to AMP (55). The activation of mucosal A2B signaling combined with the repression or deletion of epithelial ENT2 dampens mucosal inflammation (56). Another study also showed that elevations of adenosine protect from liver injury after the genetic deletion or inhibition of Ent1 *via* A2B signaling in liver ischemia and reperfusion models (57).

HYPOXIA AND INFLAMMATION IN LUNG INJURY

Hypoxia and inflammation frequently occur in pathogenic conditions such as cancer, inflammatory bowel diseases, ischemia/reperfusion injury, and inflammatory lung diseases (58). Hypoxia-inducible factors (HIFs) are crucial in the responses mediating the crosstalk between hypoxia and inflammation. Hypoxia-inducible factors (HIFs) have a central role in regulating tissue adaptation to low oxygen conditions. HIFs belongs to αβ-heterodimeric transcription factors that include HIF-1α, HIF-2α, and HIF-1β/ARNT subunits. When oxygen is abundant, HIF-1α or HIF-2α binds to the von Hippel-Lindau (VHL) gene product, a part of the E3 ubiquitin ligase complex, and result in proteasomal degradation (59–61). HIFα and VHL binding are related to the hydroxylation of HIFα proline residues, which rely on prolyl hydroxylases (PHDs) and factor-inhibiting HIF (FIH) (60, 61). Under hypoxia, HIFα subunits can not be hydroxylated as efficiently due to the lack of oxygen as a substrate for PHDs, which results in the stabilization of HIF-1α and HIF-2α. Once stabilized, HIFα translocates to the nucleus and binds to HIF-1β to form a complex, and in turn bind to hypoxia-responsive elements (HRE) of the promoter region in the target genes for start transcriptional regulation (46, 62, 63). Most of the HIFs target genes are related to metabolism, proliferation, oxygen transport, and other processes important for hypoxia adaptation (64). HIF stabilization is demonstrated in inflammatory conditions and diseases, such as lung injury, inflammatory bowel disease, and ischemia-reperfusion injury through various mechanisms (**Figure 2**) (3, 58, 65). Tissue metabolism in inflammatory disease has higher local oxygen demand, which induces tissue

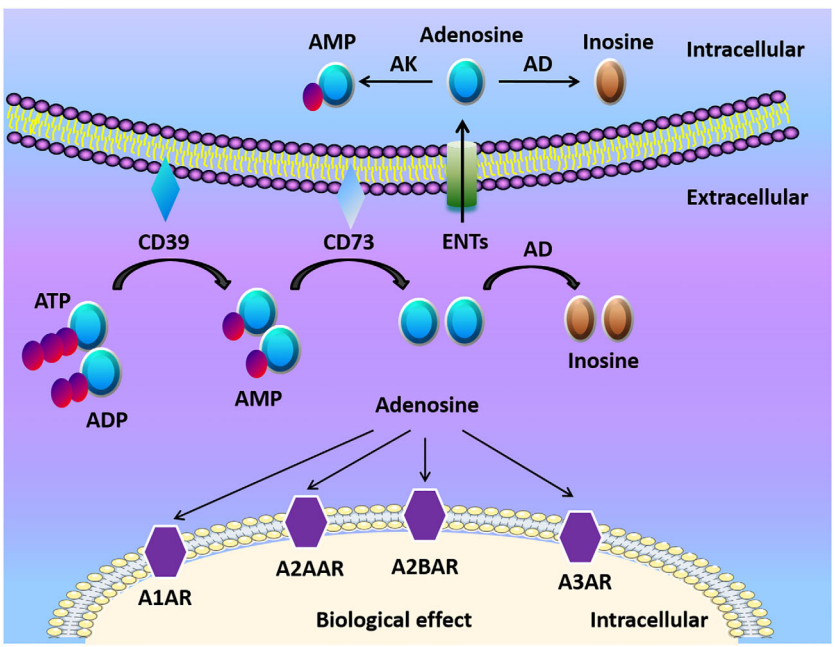

FIGURE 1 | Adenosine biogenesis and signaling. ATP and ADP are the main resources of extracellular adenosine. ATP and ADP are dephosphorylated to AMP on the cell surface by Ecto-nucleotide triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73) dephosphorylates AMP to adenosine. Adenosine activates adenosine receptors (A1AR, A2AAR, A2BAR, A3AR) and plays a crucial role in different cells and organs. Adenosine can be transported into the cell by equilibrative nucleoside transports (ENTs), or be transformed to inosine *via* CD26-bound adenosine deaminase (ADA) at the cell surface. Under normoxic conditions, adenosine has a high affinity with adenosine receptors and ENTs. Under hypoxia conditions, the release of extracellular ATP/ADP increased. Finally, HIFs enhanced the release of extracellular adenosine and adenosine receptors, which modulates tissue barriers and inflammatory response.

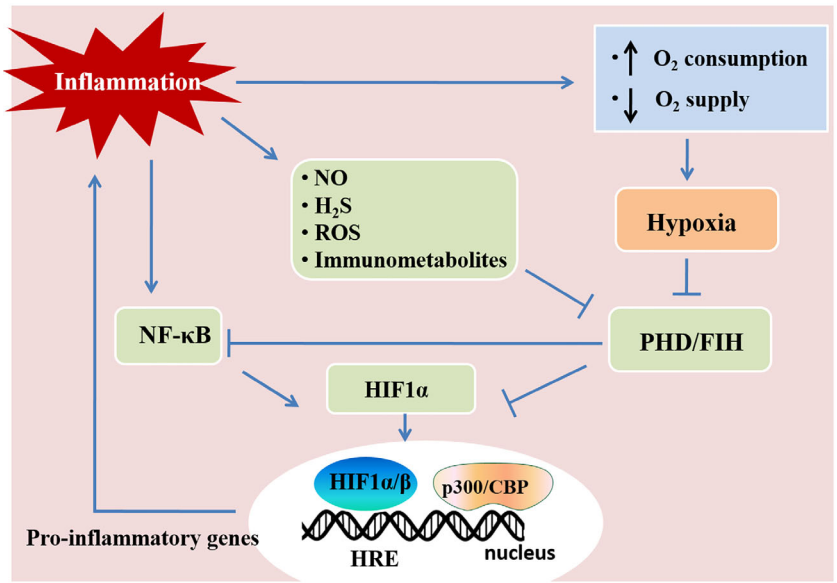

FIGURE 2 | Hypoxia and inflammation. Inflammation and hypoxia are co-incidental events in several pathological conditions. Inflammatory stimuli, such as cytokines, bacterial products, and hypoxia, activate the nuclear factor-κB (NF-κB) pathway. The activation of NF-κB enhances the transcription of HIF-1α mRNA and promotes HIF activity. Inflammatory mediators, such as nitric oxide (NO), hydrogen sulfide (H_2S), reactive oxygen species (ROS), and immunometabolites also control HIF activity in immune cells, which regulates immunity and inflammation. Activated HIF-1α translocates to the nucleus and promotes the transcription of pro-inflammatory genes by associating with HIF-1β and the cofactor p300/CBP. This figure is adapted from Regulation of immunity and inflammation by hypoxia in immunological niches; Cormac T. Taylor and Sean P. Colgan, *Nature Reviews Immunology;* 17, pages 774–785(2017) (22).

hypoxia. Additionally, the supply of oxygen decreased due to the shortage of tissue blood supply in trauma, ischemia, and vascular occlusion disease, which aggravate tissue inflammation (46, 66). Moreover, cytokines (e.g., IL-1, IFN-β, TNF-α) released during inflammation have an impact on HIF-1α expression (67, 68). The hypoxic environment in solid tumors and during ischemia/reperfusion activates NF-κB, which is a crucial transcription factor regulating inflammation and immune response (69–71). Therefore, hypoxia and inflammation usually occur simultaneously during pathogenic conditions, and they are closely linked to each other.

Acute Respiratory Distress Syndrome

Recently, increasing research effort has provided convincing evidence of the link between hypoxia and inflammation in ARDS (72–75). For example, HIF-1α is stabilized under normoxic conditions by mechanical stretch of alveolar epithelial cells *in vitro* and in ventilation-induced lung injury (VILI) in mice *in vivo* (72). The normoxic stabilization of HIF-1a by mechanical stretch could be explained by the inhibition of succinate dehydrogenase (SDH). Functionally, HIF-1α stabilization dampens lung inflammation through the regulation of glucose metabolism in alveolar epithelial cells, because only mice with alveolar epithelial cell-specific deletion of HIF-1α show profoundly increased lung inflammation and pulmonary edema (72). The protective effect of HIF-1α in alveolar epithelial cells has also been demonstrated in acute cobalt-induced lung injury models as more neutrophilic infiltration and Th1 cytokines were observed in alveolar epithelial-specific HIF-1α-deficient mice (76). Additionally, HIF-2α activation improved endothelial adherens junction integrity in endotoxin-mediated injury through increasing its target gene vascular endothelial protein tyrosine phosphatase (VE-PTP) (77). Furthermore, the pharmacologic activator of HIF, dimethyloxalylglycine (DMOG), protects the lung alveolar epithelium during murine VILI and LPS induced acute lung injury *via* enhancement of glycolysis (72, 78). Another study showed that DMOG treatment attenuates Fas Ligand (FasL)-induced apoptosis in MLE-12 cells *in vitro* and dampens lung inflammation, and histopathological changes intratracheal FasL induced lung injury in mice *in vivo* (79). These studies suggests that pharmacological HIF activator could offer lung protection during ARDS *via* maintaining alveolar epithelial and endothelial functions during injury.

Viral infection-induced ARDS has been the center of attention because of the recent pandemic of COVID-19. Influenza virus infection is one of the most studied models for viral pneumonia (80–83). Several studies have shown a close relationship between hypoxia and inflammation in viral infection-induced ARDS. For example, respiratory syncytial virus infection in mice results in the stabilization of HIF-1α in an oxygen-independent manner (84). Besides, earlier studies indicated that influenza A (H1N1) virus infection could induce HIF-1α nuclear translocation but did not change its expression levels in A549 cells *in vitro* (85). A recent study indicated that H1N1 infection stabilizes HIF-1α under normoxic conditions in

A549 cells *in vitro* and in murine models of H1N1 mediated viral pneumonia *in vivo* (86). The normoxic stabilization of HIF-1α is dependent on the inhibition of proteasome function and decreasing the expression of factor inhibiting HIF-1 (FIH-1) (86). Moreover, influenza A virus (IAV) infection-induced acute lung injury (ALI) also results in hypoxia, and further contribute to the stabilization of HIF-1α in mouse lung tissue (87). Functionally, alveolar epithelial type II cell-specific deficient *Hif1a*$^{fl/fl}$ SPCCre mice showed increased lung inflammation and mortality during IAV infection *in vivo* (87). Mechanistically, HIF-1α deficiency promotes influenza A virus replication in A549 cells *in vitro via* reducing glycolysis and enhancing autophagy (87). The functional role of HIF in SARS-CoV-2 infection associated ARDS needs to be further investigated.

Chronic Lung Injury

IPF is one of the most common and severe forms of interstitial lung disease (88). IPF patients suffer from an impaired pulmonary gas exchange and chronic arterial hypoxemia (89). The important role of hypoxia and HIFs on fibroblast proliferation and differentiation has been studied extensively (90–92). Besides the direct impact on fibroblasts, hypoxia is regarded as one of the potent stimuli for the production of proinflammatory cytokines. For example, protein kinase C (PKC) activation promotes the expression of TNF-α and IL-1β in the pulmonary artery under hypoxic conditions (93). Additionally, vascular endothelial growth factor (VEGF), a known target gene of HIF, is an angiogenesis factor with proinflammatory, permeability-inducing roles in murine bleomycin-induced pulmonary fibrosis (94). Furthermore, HIF-1α stabilization has been observed in alternatively activated macrophages in a murine model of bleomycin-induced pulmonary fibrosis and HIF-1α inhibition in macrophages inhibits the expression of profibrotic mediators including IL-7 and CXCL1 (95). However, the involvement of hypoxia signaling in other subtypes of immune cells during IPF has yet to be elucidated.

Inflammation and hypoxia are also tightly linked in COPD, including chronic bronchitis and emphysema. For example, cigarette smoking significantly increases inflammation mediators expression, such as IL-6, IL-8, and TNF-α (96). These factors contribute to the activation of hypoxia response genes (including HIFs, NF-κB) and promote the development of COPD in rats (97). HIFs are overexpressed in the lung tissue of COPD patients (98) and HIF-1α level is positively correlated with the severity of COPD in patients (99). HIF-2α, on the other hand, has been shown to be decreased in lung tissue from emphysema patients compared to healthy control (100). Furthermore, endothelial cell-specific deletion of HIF-2α in mice results in emphysematous changes in the lung, which was exaggerated by the treatment of SU5416, a vascular endothelial growth factor receptor 2 (VEGFR2) inhibitor (100). On the other hand, endothelial-specific overexpression of HIF-2α in mice was protected from emphysema (100), suggesting therapeutic activation of HIF-2 α as a treatment for emphysema.

Hypoxia is frequently encountered in patients suffering from severe asthma or acute exacerbation (101). How hypoxia and HIFs influence allergic airway inflammation has been studied extensively. An earlier study suggested that HIF-1α is stabilized in lung tissues from asthmatic patients and in a murine model of allergic airway inflammation induced by ovalbumin sensitization (102). This study also demonstrated that deficiency in HIF-1β significantly dampens allergic airway inflammation and reduced ovalbumin-specific antibodies in mice (102). Consistently, HIF-1α antagonist YC-1 reduced airway hyperresponsiveness and lung inflammation in a murine model of asthma (103, 104). Besides its global impact on allergic airway inflammation, the functional role of HIF-1α in different subsets of immune cells has also been investigated in asthma. For instance, myeloid-specific deletion of HIF-1α in mice results in reduced airway hyperresponsiveness (AHR), and HIF-1α deficient eosinophils show reduced chemotaxis (104). Furthermore, a recent study indicates that exposure to 3% oxygen leads to increased T helper type 2 cells (Th2) cytokine expression in $CD8^+$ T cells and adoptive transfer of these cells exaggerated AHR and lung inflammation in the ovalbumin model of murine allergic airway disease (105). Additionally, HIF-1α inhibition reduced Th2 cytokines expression in $CD8^+$ T cells upon hypoxia exposure, and the adoptive transfer of HIF-1α deficient $CD8^+$ T cells underwent hypoxia attenuates AHR and airway inflammation in mice (105). In summary, HIF-1α is important for the development of AHR and airway inflammation by modulating immune cell chemotaxis and function. However, the detailed mechanism, such as the identification of HIF target genes in specific immune cells during asthma, needs to be further investigated.

ADENOSINE AT THE INTERPHASE OF HYPOXIA AND INFLAMMATION IN LUNG INJURY

In the past decades, studies have provided ample evidence that hypoxia signaling is tightly linked with adenosine signaling (46, 58, 106–112). Previous studies showed that hypoxic condition or inflammation contributes to the accumulation of extracellular ATP/ADP due to the damage in the cell membrane (3, 23, 27, 113–115). The increased level of extracellular ATP and ADP is essential for the generation of extracellular adenosine, which is a key mediator of inflammatory responses (116, 117). It has been demonstrated that HRE in the promoter of CD73 gene is crucial for HIF-1α mediated expression in epithelial cells under hypoxic conditions, and the inhibition of HIF-1α decreases the hypoxia-inducible CD73 expression (118). Besides HIF-1α, transcription factor Sp1 is also involved in the transcription of CD39 under hypoxia conditions, and its protective effect has been demonstrated during cardiac and hepatic ischemia (29, 46, 119). Moreover, A2AAR has been identified as a target gene of HIF-2α in human lung endothelial cells (120), while A2BAR has been identified as a target gene of HIF-1α (121, 122). The links between HIF and adenosine are not only through regulation of ectonucleotidases and adenosine receptors but also *via* equilibrative nucleoside transporters (ENTs) and its G-protein-coupled receptors. For example, HIFs are implicated in the repression of ENT1 and ENT2 (53, 123) and abolish the conversion of adenosine to AMP by adenosine kinase in cells

(46, 55). The close relationship between hypoxia and adenosine signaling in acute and chronic lung injury have been established during the past decades (**Figure 3**).

Extracellular Adenosine Generation

Several studies suggest that adenosine level increases following hypoxia exposure in animal studies (126) and in human studies (127, 128). *In vitro* cell culture experiments and *in vivo* animal studies indicated that endogenous adenosine generation inhibits neutrophil accumulation during hypoxia (129). Particularly, CD39 deficient mice show an increased level of MPO in colon, lung, kidney, and liver after 4 h of exposure to hypoxia (8% O2) compared to wild-type mice. Pharmacological inhibition or genetic deletion of CD73 in mice leads to a similar phenotype as CD39 deficient mice, suggesting the importance of extracellular adenosine generation in hypoxia-induced inflammation. Moreover, short term exposure to hypoxia increases plasma levels of adenosine, attenuates pro-inflammatory cytokine release, and results in an elevated level of IL-10 during experimental endotoxemia models in humans (130). Extracellular adenosine levels increase after the mechanical ventilation in mice or stretched pulmonary epithelial cells (106, 112). Pharmacological inhibition or genetic deletion of CD39 or CD73 in mice leads to severe lung inflammation with mechanical ventilation, suggesting the protective effect of adenosine (106). The relationship between HIF and adenosine in ARDS during viral pneumonia has not been clearly demonstrated yet. Nucleotide ATP and adenosine in BALF have been shown to be increased after the infection of influenza A virus in mice (131, 132). However, adenosine levels and pathogenesis of ALI did not show any difference between WT and CD73-knockout mice after the infection of influenza A virus. Therefore, CD73 is not considered as one of the crucial factors for the development of influenza-induced ALI (133).

Cellular stress and damage induce the generation of adenosine in lung tissue of patients with chronic lung disease. For example, the hypoxic-adenosinergic pathway is activated in IPF patients with pulmonary hypertension (PH), as marked by increased expression of HIF-1α, adenosine, adenosine A2B receptor, CD73, and ENT1 (124). Other studies showed that adenosine levels are increased in the serum, lymphocytes, and erythrocytes in healthy smokers compared to healthy non-smokers and continue to increase with the severity in COPD patients (134). The same study also demonstrated that patients with higher levels of adenosine tend to have reduced forced expiratory volume in one second (FEV1), suggesting a potential functional link (134). Furthermore, adenosine signaling is significantly enhanced in COPD as represented by increased CD73 activity and adenosine receptor levels in lung tissue from patients with COPD or in murine model of emphysema (135, 136). Adenosine signaling is also enhanced in asthma, and consequently, a high level of adenosine induces airway hyperresponsiveness and bronchoconstriction and promotes human mast cells to release allergen-induced mediators (137).

Adenosine Receptors and Signaling

Adenosine A_1 Receptor

A1AR has diverse roles in lung injury. For example, A1AR deficient mice have increased susceptibility to LPS-induced acute lung injury with increased PMN recruitment and microvascular permeability (138). The same study indicated that pretreatment of A1AR agonist, 2'Me-2-chloro-N6-cyclopentyladenosine, attenuates PMN recruitment and microvascular permeability. On the other hand, post-infection treatment of a combination of A1AR antagonist L-97-1 and ciprofloxacin improves the outcome of *Y. pestis* infection in rats, indicating a protective effect of A1AR (139). Furthermore, A1AR

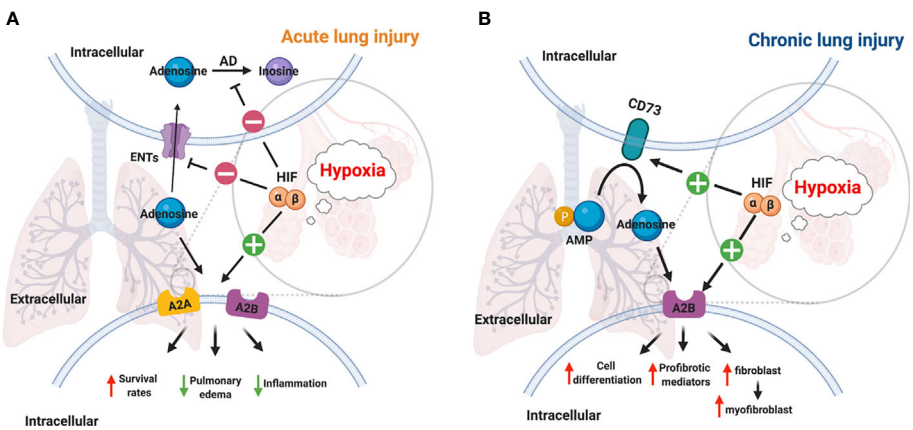

FIGURE 3 | Links between HIF and adenosine signaling in acute/chronic lung injury. **(A)** Inflammation and infection results in the stabilization of HIFs in acute lung injury (112). HIF-1a dependent inhibition of ENT1, ENT2, and adenosine kinase contributes to the accumulation of adenosine (55, 123). A2AAR and A2BAR are two adenosine receptors that are regulated by HIF-2α and HIF-1α respectively in lung tissue (109, 120, 121). Therefore, the higher level of adenosine, and the activation of its receptors reduced mortality, pulmonary edema, inflammation in acute lung injury. **(B)** The activation of the hypoxic-adenosinergic system has been investigated in chronic lung injury. CD73 and A2BAR are two hypoxia-inducible genes in patients with idiopathic pulmonary fibrosis and pulmonary hypertension (124). The upregulation of A2BAR enhances cell differentiation, produces profibrotic mediators, and promotes fibroblast to myofibroblast in chronic lung injury (95, 125).

knockout mice show significantly increased macrophage and neutrophil infiltration in the airway after influenza A/WSN/33 (H1N1) infection when compared to wild-type counterparts and daily treatment of A1AR antagonist 8-cyclopentyl-1,3-dipropylxanthine results in improved outcome (140). Besides acute lung injury and infection, the activation of A1AR has also been found on bronchial epithelial cells, and inflammatory cells, which enhanced the asthma phenotype (141). Mice with ADA deficiency experience lung injury and inflammation (142). A1AR deletion in mice exaggerated the pulmonary inflammation marked by increased expression of IL-4 and IL-13, as well as matrix metalloproteinases, suggesting a protective role of A1AR in chronic lung injury (142).

Adenosine A2A Receptor

Exposure to hypoxia (10% O_2) attenuates lung inflammation during LPS-induced lung injury in mice and A2AAR is indispensable for hypoxia-mediated lung protection (143). A2AAR expression in myeloid cells is crucial for the control of neutrophil recruitment to the lung injury and an A2AAR specific agonist ATL202 offers lung protection in mice during LPS-induced lung injury (144). The lung protective effect of A2AAR has also been implicated in cardiopulmonary bypass-mediated lung injury. Pretreatment of A2AAR agonist CGS21680 in juvenile rats dampens inflammatory cytokines and myeloperioxidase levels in the serum as well as pulmonary edema and lung injury score during cardiopulmonary bypass –induced organ injury. Another study demonstrated that A2AAR activation induces the expression of peroxisome proliferator-activated receptors γ (PPARγ) *via* cAMP and PKA pathways in murine macrophages (145). Combining PPARγ agonist ROSI and A2AAR agonist CGS21680 significantly reduces lung pathology and the production of inflammatory cytokines in the lung during murine model of LPS-induced ALI (145). Moreover, treatment of CGS21680 after the onset of trauma/hemorrhagic shock-induced lung injury attenuates pulmonary edema and MPO levels in Sprague-Dawley rats (146). Interestingly, A2AAR has been identified as a HIF-2α target in pulmonary endothelial cells *in vitro*, implicating the crosstalk between adenosine signaling and hypoxia signaling (120). Furthermore, treatment of A2AAR agonist CGS21680 reduces inflammatory cell infiltration to the airway in murine models of asthma (147). A2AAR deficient mice experience exaggerated lung inflammation and airway hyperactivity, suggesting the protective role of A2AAR in allergic airway diseases.

Adenosine A2B Receptor

A2BAR is an important link between hypoxia and adenosine signaling in acute lung injury. HIF-1α has been shown to transcriptionally induce the expression of A2BAR in murine VILI model (109, 121). For instance, genetic silence or pharmaceutical inhibition of HIF-1α dampens the expression of A2BAR in mice during VILI or alveolar epithelial cells exposed to cyclic stretch (109). A2BAR-dependent adenosine signaling offers lung protection during endotoxin-induced ALI in mice by potentiating the regulatory T cell population (148). Furthermore, hypoxia-induced vascular leakage also exaggerates in siRNA-mediated knockdown of A2BAR or A2BAR deficient mice (149). Furthermore, HIF-1α-dependent induction of netrin-1 attenuated neutrophil transmigration and dampens inflammation through A2BAR at pulmonary and colon mucosal surface (150), suggesting another layer of complexity in the crosstalk between HIF and adenosine signaling.

In chronic lung injury, hypoxia potentiates the function of adenosine and promotes the production of IL-6, and induce the differentiation of fibroblasts to myofibroblasts by increasing adenosine A2B receptor expression in human fibroblasts (125). Furthermore, adenosine deaminase-deficient mice have higher expression of A2BAR and exhibit progressive pulmonary fibrosis and respiratory distress (151). The crosstalk between hypoxia and adenosine signaling has been established in the murine model of IPF (95). For example, HIF-1α inhibition *via* the treatment of 17-DMAG results in reduced pulmonary fibrosis and A2BAR expression in the late stages of murine bleomycin-induced lung fibrosis *in vivo* (95). Additionally, HIF-1α inhibition along with A2BAR deletion or pharmacological inhibition result in disruption of alternatively activated macrophages differentiation and IL-6 production *in vitro* (95).

Of note, while A2AAR and A2BAR reduce mortality, pulmonary edema, and inflammation in acute lung injury, A2BAR enhances cell differentiation, produces profibrotic mediators, and promotes fibroblast to myofibroblast differentiation in chronic lung injury. The differential role of A2BAR could possibly be stemming from the different impacts of downstream signaling in acute or chronic lung injury. As mentioned above, A2AAR and A2BAR activation lead to the activation of cAMP and PKA pathway (32–35). The cAMP-CREB axis is important for the maintenance of endothelial integrity and the attenuation of lung inflammation during endotoxin-induced lung injury in mice (152). The protective effect of cAMP in LPS-induced endothelial permeability is mediated through PKA (153). cAMP synthesis and PKA activity are inhibited in oleic acid-induced lung injury, and the treatment of hydroxysafflor yellow A enhances the cAMP/PKA pathway and dampened lung inflammation in mice (154). Furthermore, pretreatment of phosphodiesterase antagonist PTX enhances cAMP signaling and results in the attenuation of lung injury during cecal ligation and puncture in mice (155). These studies suggest a protective role of cAMP and PKA during acute lung injury. In chronic lung injury, cAMP and PKA regulate hypercontractility in human airway smooth muscle cells (156) and phosphodiesterase inhibitors, which prevents the breakdown of cAMP, are currently being studied as a treatment for asthma (157). In addition, dibutyryl-cAMP treatment increases endogenous cAMP levels, enhances PKA signaling *in vitro*, and blocked myofibroblast differentiation *in vivo* (158). Other cAMP elevating agents also inhibits the proliferation and collagen production in pulmonary fibroblasts (159). Thus, the divergent function of A2BAR in acute and chronic lung injury might not be based on the downstream activation of the cAMP and PKA signaling pathway. Other factors could contribute to the response to cAMP activation as lung fibroblasts from pulmonary fibrosis patients has a deficiency

in the phosphorylation of cAMP response element-binding protein (160). Future studies are needed to elucidate the signaling mechanism of A2BAR mediated responses in pulmonary injuries.

Adenosine A3 Receptor

A3AR is also expressed in the lung and several previous studies have indicated the functional role of A3AR in lung injury. The protective role of A3AR in lung ischemia/reperfusion injury has been demonstrated by an early study in which the pretreatment of A3AR agonist IB-MECA attenuated alveolar injury and apoptosis during lung ischemia and reperfusion injury of isolated cat lung *ex vivo* (161). The protective role of A3AR is further supported as pretreatment of IB-MECA offers lung protection during lung ischemia/reperfusion injury in cat *in vivo* (162). In addition, A3AR agonist CI-IB-MECA pretreatment alleviates lung ischemia/reperfusion injury in mice, and the protective effect is abolished in mice with genetic deletion of A3AR (163). Besides lung ischemia/reperfusion injury, the protective role of A3AR has also been indicated in LPS-induced lung injury. Indeed, A3AR deficient mice showed exaggerated PMN infiltration after LPS inhalation and pretreatment of CI-IB-MECA attenuates the inflammatory responses and injury (164). Furthermore, A3AR activation is associated with mast cell degranulation and airway hyperreactivity. For example, selective activation of A3AR *via* IB-MECA results in the release of histamine in mast cells *in vitro* and nebulizer treatment of IB-MECA in mice results in mast cell degranulation in the lung in wild type mice but not in A3AR deficient mice (165). Adenosine administration results in airway responsiveness in mice and mice with A3AR deficiency show attenuated responses marked by reduced mast cell degranulation and neutrophil infiltration (166). Other studies also demonstrate the contribution of A3AR in chronic airway inflammation (167, 168).

Adenosine Metabolism

Besides the impact on adenosine receptors, HIF-1α dependent repression of ENT1 and ENT2 decreases adenosine uptake and increases extracellular adenosine, which dampens neutrophil accumulation and protects vascular barrier during hypoxia in endothelia and epithelia (123). HIF-1α-dependent repression of adenosine kinase leading to increased extracellular adenosine attenuates hypoxia-induced vascular leak in murine models of sepsis or ALI (169). In addition, adenosine deaminase activity, ADA2 in particular, is significantly reduced in serum from COPD patients and smokers when compared to non-smokers (134), which could further explain the increased level of adenosine in COPD patients.

THERAPEUTIC TARGETING OF ADENOSINE

Targeting Hypoxia Signaling

Direct therapeutic targeting of the hypoxia signaling pathway could profoundly modulate the adenosine signaling pathway. Pharmacologic compounds have been developed for normoxic stabilization of HIFs by functioning as inhibitors of PHDs. Several preclinical studies show that these compounds can be given to animals that are kept under normoxic conditions, and result in robust stabilization of HIFs (170, 171). In line with this concept, preclinical studies have shown that pretreatment with the HIF activator dimethyloxalylglycine (DMOG) is associated with attenuated organ injury in the heart, lungs, or kidneys (72, 172, 173). Moreover, recently, several pharmaceutical companies have developed HIF activators as orally available compounds and several ongoing clinical trials have used them in patients for the treatment of anemia associated with chronic kidney disease. These compounds include roxadustat (FG-4592, sponsored by FibroGen, Astellas, & AstraZeneca), vadadustat (AKB-6548, sponsored by Akebia), and daprodustat (GSK-1278863, sponsored by GlaxoSmithKline). Based on phase 3 clinical trials showing efficiency in increasing hemoglobin levels in patients with anemia associated with renal insufficiency (174, 175), roxadustat has been approved for treating chronic kidney disease-related anemia in China and is currently in phase 3 clinical trials in the United States. In the meantime, several phase 2 clinical trials indicated that oral vadadustat is safe and effective as a treatment for anemia in patients with non-dialysis-dependent chronic kidney diseases (176, 177), and in patients receiving hemodialysis previously received erythropoiesis-stimulating agents (178). Currently, vadadustat is evaluated by a randomized, double-blinded and placebo-controlled phase 2 clinical trial as a treatment of COVID-19 associated ARDS (**Table 1**) (180). These oral available HIF activators could potentially be efficient for enhancing adenosine signaling pathways in patients for the prevention of acute lung injury. On the other hand, HIF inhibitors could potentially inhibit adenosine signaling as a therapeutic approach for chronic lung diseases. Currently, HIF-2α inhibitors such as PT2385 and PT2977 have been investigated by clinical trials mainly as novel therapeutic approaches for renal cell carcinoma (181). However, HIF-1α specific inhibitor has yet to be developed for clinical use, which will be crucial for the inhibition of adenosine signaling in chronic lung diseases.

Targeting Adenosine Receptors

Adenosine signaling could potentially be targeted for lung protection during acute lung inflammation *via* direct administration of adenosine or utilizing specific adenosine receptor agonists in both preclinical and clinical settings (182, 183). Several preclinical studies have indicated that direct administration of adenosine attenuates lung injury (184, 185). The safety of adenosine administration has also been supported by previous clinical studies (186, 187). However, due to the short half-life of adenosine *in vivo*, adenosine analogs might be a more feasible option. Adenosine receptor agonists have been developed for preclinical and clinical use (188). For example, pretreatment of A2AAR agonist ATL202 inhibits LPS-induced PMN recruitment, reduced the release of inflammatory cytokines in the lung, and reduced vascular leakage in mice (144). A2AAR agonist GW328267C improves lung function in three models of ALI (HCl instillation 1 h, LPS instillation 16 h, and live

TABLE 1 | Clinical trials targeting adenosine signaling in lung diseases.

Drug (Company)	Target	Status	Target disease	Clinical trial gov identifier	References
GW328267X	A_{2A} adenosine receptor agonist	Completed Phase 1	Acute lung injury	*NCT01640990*	
PBF-680	A1 adenosine receptor antagonist	Completed Phase 2	Asthma	*NCT01939587*	
PBF-680	A1 adenosine receptor antagonist	Recruiting Phase 2	Asthma	*NCT02635945*	
PBF-680	A1 adenosine receptor antagonist	Completed Phase 1	Asthma	*NCT01845181*	
PBF-680	A1 adenosine receptor antagonist	Completed Phase 1	Asthma	*NCT02208973*	
PBF-680	A1 adenosine receptor antagonist	Recruiting Phase 2	Persistent, mild-to-moderate atopic asthma	*NCT03774290*	
Regadenoson	A_{2A} adenosine receptor agonist	Completed Phase4	As stress agents for myocardial perfusion imaging in asthma or COPD patients	*NCT00862641*	(179)
Dipyridamole	Equilibrative nucleoside transporter inhibitor	Recruiting Phase 2	COVID-19; SARS-CoV-2 infection	*NCT04391179*	
Dipyridamole	Equilibrative nucleoside transporter inhibitor	Recruiting Phase 2	COVID-19 pneumonia; Vascular complications	*NCT04424901*	
Vadadustat	Hypoxia-inducible factor prolyl hydroxylase (HIF-PH) inhibitor	Recruiting Phase 2	Acute respiratory distress syndrome; coronavirus infection	*NCT04478071*	

Escherichia coli instillation) in rats (189). The delivery of A2BAR-specific agonist BAY 60-6583 attenuate pulmonary edema, inhibits lung inflammation, and improves histologic lung injury in murine ALI (73, 107). Furthermore, mice treated with BAY60-6583 show attenuated oleic acid (OA)-induced ALI by inhibiting alveolar epithelial cell apoptosis (190). However, only A1AR, A2AAR, and A3AR agonists have been evaluated in the clinical setting while the safety and efficacy ofA2BAR agonists have yet to be established by clinical studies (188). The usage of adenosine receptor agonists in clinical trials related to lung injury is summarized in **Table 1**.

Adenosine receptor antagonists have been developed as treatment of chronic lung diseases in both preclinical and clinical settings. For instance, LASSBio-897 (3-thienylidene-3, 4-methylenedioxybenzoylhydrazide) can block the activity of A2AAR agonist and has anti-inflammatory and anti-fibrotic role in a mouse model of silicosis (191). Additionally, the treatment of A2BAR antagonist CVT-6883 dampens lung inflammation, reduces fibrosis, and attenuates alveolar airspace enlargement in ADA-deficient mice (192). Similarly, CVT-6883 treatment reduced inflammation and lung fibrosis in murine bleomycin-induced lung injury (192). Finally, A1AR antagonist PBF-680 has been and is currently being evaluated by several phase 1 and phase 2 clinical trials as a treatment of asthma (**Table 1**). Although adenosine receptor antagonists have been investigated for inflammatory conditions, neurodegenerative diseases, and mood disorders (193), their potential impact on chronic lung diseases needs to be further evaluated.

Targeting Adenosine Metabolism

Adenosine signaling could also be targeted *via* modification of adenosine metabolism. For instance, inhibition or deletion of ENT1/2 elevates extracellular adenosine levels in lung tissue and improves pulmonary function by activating A2AAR and A2BAR receptor and preventing NLRP3 inflammasome activation in *Pseudomonas aeruginosa* infection-induced acute lung injury in mice (194). Moreover, ENT inhibitor dipyridamole treatment decreases adenosine uptake, and in turn improves vascular barrier and reduces neutrophil accumulation in acute pulmonary inflammation in preclinical studies (108, 123, 195). Along the same line, dipyridamole is currently investigated by several clinical trials as a treatment for COVID-19 and associated vascular manifestation (NCT04391179, NCT04424901, **Table 1**). Besides targeting ENTs, ADA administration reduced lung pathology in IL-13 transgenic mice, which spontaneously develop lung inflammation, alveolar destruction, and fibrosis (196). Furthermore, PEGylated adenosine deaminase is currently employed as an enzyme replacement therapy for patients suffering adenosine deaminase severe combined immunodeficiency (197) and has lately been shown to alleviate fibrosis and inflammation in a murine model of systemic sclerosis (198). PEGylated adenosine deaminase should be further investigated as a therapeutic approach for chronic lung diseases.

CONCLUSION

Adenosine signaling is one of the most crucial mediators in the cross-talk between hypoxia and inflammation. In this review, many studies suggest that targeting hypoxia and adenosine signaling could be a promising therapeutic approach for ARDS and chronic lung diseases. However, further investigation is needed to address the knowledge gaps in the mechanism of how HIF-adenosine contributes to different disease conditions and how to target this pathway in patients. For instance, the functional link between HIF and adenosine pathway in viral pneumonia induced ARDS needs to be established, especially for COVID-19 associated ARDS. Furthermore, the functional role of

the HIF-adenosine pathway needs to be demonstrated in COPD and asthma for the development of novel therapies targeting this pathway. Pharmacological agents to modulate adenosine signalings, such as adenosine receptor antagonists and PEGylated adenosine deaminase, have been investigated in several disease conditions. However, its potential use for chronic lung diseases needs to be further evaluated. Taken together, a detailed understanding of the functional role of the HIF-adenosine axis is needed for the development of efficient and safe therapy in pulmonary diseases.

AUTHOR CONTRIBUTIONS

XL drafted the manuscript. NB assisted with the literature search. TM revised the manuscript. KZ assisted with the figures. HE revised the manuscript and provided critical advice on the structure and content of the manuscript. XY drafted and finalized the manuscript and provided critical advice on the structure and content of the manuscript. All authors contributed to the article and approved the submitted version.

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