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Trends in Immunology

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Review

Purinergic Signaling During Immune Cell Trafficking

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Migration and positioning of immune cells is fundamental for their differentiation and recruitment at sites of infection. Besides the fundamental role played by chemokines and their receptors, recent studies demonstrate that a complex network of purinergic signaling events plays a key role in these trafficking events. This process includes the release of nucleotides (such as ATP and ADP) and subsequent autocrine and paracrine signaling events through nucleotide receptors. At the same time, surface-expressed ectoapyrases and nucleotidases convert extracellular nucleotides to adenosine, and adenosine signaling events play additional functional roles in leucocyte trafficking. In this review we revisit classical paradigms of inflammatory cell trafficking in the context of recent studies implicating purinergic signaling events in this process.

The Purinergic Network: Transducing Extracellular Nucleotide and Nucleoside Signaling

Nucleotides such as ATP, UTP, GTP, and ADP and the nucleoside adenosine are well known for their fundamental intracellular roles. ATP, for example, represents an 'energy store' for virtually all cells and in addition is a basic constituent of nucleic acids and a crucial enzyme modulator. Interestingly, nucleotides and nucleosides show completely different roles when present in the extracellular compartment. Hence, liberation of ATP, UTP, UDP, ADP, and adenosine occurs in many cell types and in completely different contexts [1,2]. Any cell can passively release nucleotides and nucleosides if the plasma membrane undergoes heavy stress or damage. The main cell membrane stressors include allergens, extracellular proteases, oxygen radicals (ROIs), and shear stress forces (Figure 1) and also bacterial products and viruses. It has become clear that cells physiologically (i.e., in the presence of homeostatic conditions) release, in a regulated manner, nucleotides and nucleosides that function as extracellular signaling molecules [1,2]. Regardless of where they come from, once in the extracellular milieu nucleotides and nucleosides bind specific plasma membrane receptors needed for cell-to-cell communication named purinergic receptors. These are grouped into P2 and P1 receptors, respectively [3]. Based on their sequence and signaling properties, the G protein-coupled P1 receptors are further distinguished as four subtypes (A_1, A_{2A}, A_{2B}, and A_3), while P2 receptors form two groups comprising the metabotropic G protein-coupled P2Y receptors [4] and the ionotropic P2X receptors [5]. Nucleotides (ATP, ADP, UTP, and UDP) formed or released in the extracellular compartment bind and activate P2 receptors, while adenosine present in the extracellular milieu is an agonist for P1 receptors.

P2Y receptors are seven-transmembrane spanning receptors expressed by virtually all mammalian tissues. They have an extracellular NH_3 domain, while the COOH terminus is intracellular. Unlike P2X receptors, they are not ion channels and their activation induces the formation of various intracellular messengers and liberation of Ca^{2+} from intracellular stores. Eight P2Y receptors have been identified so far: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13,

Trends

Nucleotides and nucleosides are well known for their intracellular functions as cellular energy currency ad as building blocks for DNA and RNA. In the extracellular compartment, however, they function as signaling molecules.

Nucleotides are converted in the extracellular compartment to nucleosides via the activity of enzymatic systems such as CD39 (conversion of ATP/ADP to AMP) and CD73 (conversion of AMP to adenosine).

Extracellular nucleotide signaling via P2 receptors drives inflammatory responses and is critical for bacterial killing. By contrast, nucleoside signaling via P1 receptors dampens inflammatory responses.

Enzymatic conversion of nucleotides into nucleosides can play a critical role in modulating an immune response associated with a shift from proinflammatory P2 signaling to anti-inflammatory P1 signaling.

Purinergic signaling modulates chemokine release and the outcome of the immune response. Pro- and anti-inflammatory effects mediated by extracellular nucleotides influence tissue fate either directly or through chemokine/chemokine receptor signaling.

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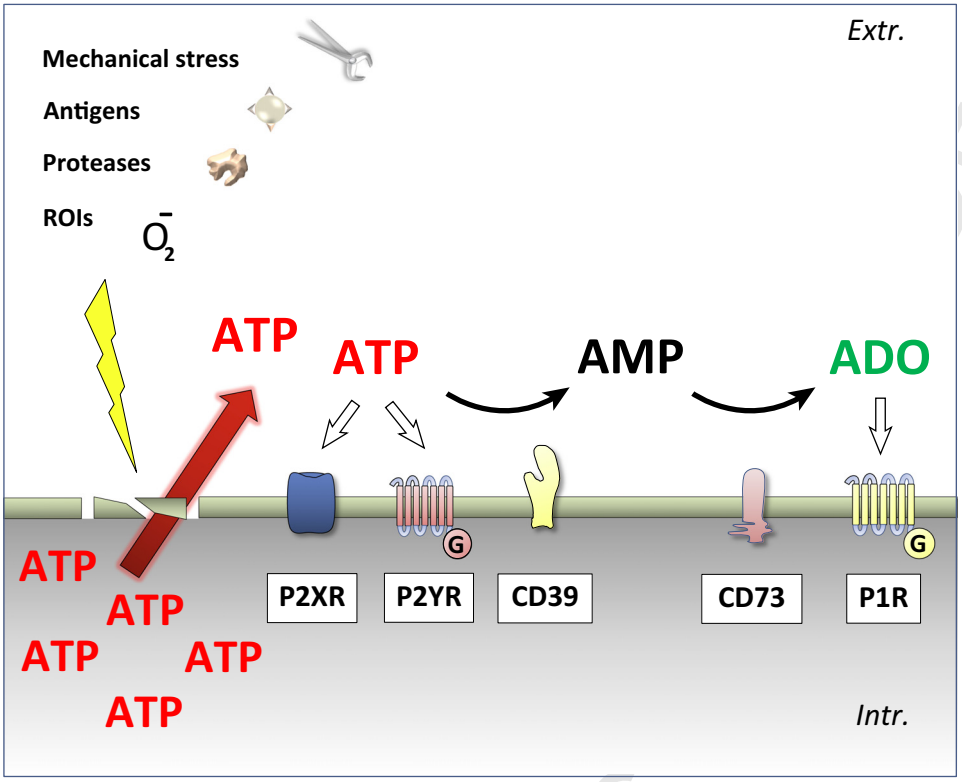

Figure 1. The Purinergic Network. A range of events including hypoxia, apoptosis, necrosis, stress due to mechanical forces, infections by microorganisms, secretion of proteases, and production of oxygen radicals (ROIs) cause cells to release nucleotides and nucleosides into the extracellular milieu. Once released extracellularly, nucleotides (ATP, ADP, UTP, UDP, etc.) behave as signaling molecules through activation of the purinergic P2 (P2Y and P2X) receptors. The extracellular presence of the nucleoside adenosine (ADO) is mainly due to generation at the cell membrane as a consequence of the enzymatic conversion of ATP to ADO by sequential activation of the ectonucleoside triphosphate diphosphohydrolase CD39 (ATP/ADP converted to AMP) and the ecto-5′-nucleotidase CD73 (AMP converted to adenosine). ADO is an agonist at P1 purinergic receptors, which are represented by four subtypes: A_1, A_{2A}, A_{2B}, and A_3.

Glossary

Chemokinesis: random cell movement in the absence of a chemoattractant gradient.
Chemotaxis: oriented movement of cells or organisms in response to chemicals that attract (positive chemotaxis) or repel (negative chemotaxis) them.
CXCL8 (IL-8): chemokine secreted by various cells (macrophages, endotheliocytes, epithelial and smooth muscle cells); binds to the CXCR1 and CXCR2 receptors.
CXCL12 (stromal-derived factor-1): binds to CXCR4 and CXCR7 receptors regulating cell trafficking under normal and neoplastic conditions. Particularly relevant for tumor growth and metastatic diffusion.
Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10): also called CDw156 or CD156c; a metallopeptidase cleaving membrane protein at the cell surface.
F1 ATPase: also named F(1)/F(0)-ATP synthase; a plasma membrane enzyme with the ability to produce extracellular ATP but also to hydrolyze ATP into ADP.
Fractalkine: also known as CX_3CL1; a chemokine binding to CX3CR1 and attracting T cells, monocytes, and NK cells. CX_3CL1 and CX3CR1 also stimulate the cytolytic activity of NK and $CD8^+$ cytotoxic T lymphocytes.
Monocyte chemotactic protein 1 (MCP-1): also referred to CCL2 or small inducible cytokine A2; involved in diseases such rheumatoid arthritis, atherosclerosis, and psoriasis that are characterized by heavy monocyte tissue infiltration.
Regulated on activation, normal T cell expressed and secreted (RANTES): also called CCL5; binds to CCR5 and is chemotactic for monocytes and memory T lymphocytes.

and P2Y14 [4]. The first five couple to Gαq family proteins and activate phospholipase C-β (PLC-β) isozymes; the ATP-activated P2Y11 receptor also couples to Gαs activating adenylyl cyclase. P2Y12, P2Y13, and P2Y14 couple to the Gαi/o family and inhibit adenylyl cyclase activity. The abundance of P2Y subtypes is paralleled by the variety of endogenous ligands able to bind them [4]: ATP, ADP, UTP, UDP, UDP-glucose, and other UDP-sugars as well as uridine adenosine tetraphosphate (Up4A). While ADP activates the P2Y1, P2Y12, and P2Y13 subtypes, ATP is an agonist at P2Y2 and P2Y11. Uridine nucleotides (UTP and UDP) activate P2Y2, P2Y4, and P2Y6. P2Y14 is activated by UDP, UDP-glucose and other UDP-sugars [4]. Involvement of some P2Y receptor subtypes (P2Y2, P2Y6, and P2Y12) in host defense and inflammation has been ascertained [6,7].

Receptors for extracellular ATP and ADP are named P2X receptors. These are plasma membrane channels selective for monovalent and divalent cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) that are directly activated by the agonist [5]. Seven different subunits have been identified so far and numbered from 1 to 7 (P2X1R–7R). They have two transmembrane domains, a large extracellular loop with the ability to bind ATP, which is an agonist at all subtypes, and intracellular N and C termini. Opening of the receptor channel by the agonist induces transmembrane ion fluxes

leading to plasma membrane depolarization, activation of Ca^{2+}- and K^{+}-dependent enzymatic cascades as in the case of P2X7-mediated pro-caspase 1 activation, and subsequent pro-IL-1 and pro-IL-18 cleavage and secretion [8,9].

In the case of ATP and ADP, nucleotide metabolism produces adenosine, which activates P1 receptors [10,11]. Four ectonucleotidase groups [i.e. ectonucleoside triphosphate diphosphohydrolases (NTPDases), ecto-59-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterase, and alkaline phosphatases] [12] have been identified and have the ability to degrade ATP and its metabolites, eventually leading to adenosine production [13]. Plasma membrane receptors for extracellular adenosine are named P1 receptors. The four identified subtypes (A_1, A_{2A}, A_{2B}, and A_3) share a common membrane topology, with seven-trans-membrane spanning domains, and all couple to G proteins [14]; their activation induces concentration changes in the intracellular second messenger cAMP and, with the A_{2B} subtype, Ca^{2+} release from intracellular stores. Adenosinergic receptors modulate many biological functions, ranging from heart rate, vascular tone, and neuron excitation to the immune response. Concerning the latter, they mainly activate anti-inflammatory pathways [15]. Adenosine is then sequestered or degraded extracellularly, thus causing termination of P1-mediated signaling [16].

Besides 'stress-induced' nucleotide release, different cell types (neurons, platelets, endothelliocytes, lymphocytes, epithelial cells) can release ATP and ADP through specialized plasma membrane molecules (connexin hemichannels, pannexin channels, the P2X7 receptor, ABC transporters, ATP-conducting anion channels) [17,18]. A recently identified system allows accumulation of ATP in intracellular vesicles expressing the vesicular nucleotide transporter (VNUT) SLC17A9 and release of the nucleotide by exocytosis [19]. Nucleotides and nucleosides activate immune cells, helping them to fight microbes [20]. However, uncontrolled ATP release has been shown to induce excessive secretion of proinflammatory mediators (prostaglandins, ROIs, chemokines, proinflammatory cytokines) and massive recruitment of immune cells, which is detrimental for tissue integrity as it exacerbates inflammation [21,22]. To protect tissues, particularly from immune-mediated tissue damage, adenosine is generated and in many cases dampens inflammation through P1 receptor stimulation [15]. Therefore, purinergic signaling represents the result of the activity of a complex and heterogeneous 'molecular machinery' comprising nucleotide/nucleoside transporters, plasma membrane P1 and P2 receptors, and nucleotide-degrading enzymes (CD39, CD73) (Figure 1) cooperating to form the inflammatory microenvironment in which leukocytes act [12,13]. Accordingly, responses mediated by immune cells are heavily influenced by, and in many cases dependent on, the purinergic signaling predominating in a particular tissue context [16]. In particular, P1 and P2 receptors frequently show opposing effects in terms of modulation of the immune response, and shifting the balance from purinergic P2-mediated signaling (mostly proinflammatory) to adenosine-mediated P1 signalling (prevalently anti-inflammatory) or vice versa may have important consequences on the immune outcome. In other words, stimulation of adenosine receptors exerts beneficial effects by down-modulating inflammation and thus protecting tissues from immune-mediated damage [15] while activation of P2 receptors by ATP can exacerbate and prolong tissue inflammation [16].

Recent findings implicate a role for purinergic signaling in producing and modulating the **chemotaxis** (see Glossary) of leukocytes, since autocrine and paracrine stimulation of P1 and P2 receptors substantially contributes to cell polarization and leucocyte migration [23–26]. Moreover, nucleotides and nucleosides contribute to regulating the secretion of chemokines, which is fundamental in attracting immune cells to the site of infection [27–30]; in most cases, P1 receptors inhibit or reduce chemokine secretion while P2 receptor stimulation increases it.

Chemotactic Properties of Nucleotides and Nucleosides

To respond to an infection, immune cells have to reach tissue sites where invading microorganisms are present. Therefore, leukocytes are programmed to exit the circulation and move toward epicenters of infection/inflammation, guided by chemical gradients of various stimuli. The oriented migration of cells inside chemical gradients is termed 'chemotaxis' and is evoked by so-called 'chemoattractants', a large and heterogeneous group of chemicals including both soluble molecules produced by the host and non-self constituents [*N*-formyl peptides, lipopolysaccharide (LPS)] from invading microorganisms [31]. The former include platelet-activating factor (PAF), complement protein 5a (C5a), leukotriene B_4 (LTB_4), lipoxin A_4 (LXA_4), and the large chemokine family [31,32].

The chemotactic process involves a complex sequence of coordinated changes that are made possible by the expression on the cell membrane of chemotaxis receptors that, on binding with their ligands, allow cells to proceed in a chemical gradient. Convincing data obtained by pharmacological inhibition of various purinergic subtypes or by knockout mice for single P1 or P2 receptor subtypes clearly show that nucleotides and nucleosides function as chemoattractants for leucocytes, particularly phagocytes; an example is the reduction of chemotaxis in monocytes/macrophages in the presence of the enzyme apyrase (which degrades extracellular ATP) and in animals lacking the P2Y receptor subtype ($P2Y2^{-/-}$ mice). Moreover, pharmacological inhibition or stimulation of single or multiple purinergic receptor subtypes has also been important in understanding extracellular nucleotide- and nucleoside-induced chemotaxis [22,33,34]. Another interesting finding is that purinergic receptors contribute to both positive excitatory signals (needed at the front of the cell) and the inhibitory signals that must be provided to the rest of the plasma membrane, particularly to the back of a migrating cell to allow its navigation in a chemotactic gradient [38]. Therefore, the concerted participation ATP/ADP-activated P2 receptors, ectonucleotidases (to generate adenosine), and P1 receptors is needed to obtain efficient chemotactic migration.

Initial studies documented the chemotactic effects of pharmacologically added nucleotides (ATP, ADP, UTP, UDP-glucose in immune cells) [24,26,36], but it was also realized that chemotactic stimuli released by microorganisms (fMLP, LPS) or formed during the onset of inflammation (C5a) evoke the release of nucleotides that enhance or cooperate in the production of robust cytokine secretion and efficient chemotactic migration, thus increasing the likelihood that invading microorganisms will be reached and eliminated [23]. The contribution of autocrine purinergic signaling to leukocyte migration is not ancillary since, for example, enzymatic degradation of secreted ATP by apyrase impairs monocyte migration and recruitment in a mouse model of C5a-induced peritonitis [38].

Besides defense against invading microorganisms, phagocytes have the fundamental role of eliminating dead cells and tissue debris. Although it is intuitive that damaged tissues or necrotic cells release ATP, it is less intuitive that apoptotic cells also release nucleotides that function as a 'find-me signal' inducing oriented migration of phagocytes and thus favoring apoptotic body clearance [23,39,40]. To this purpose, the participation of pannexin-1 channels links ATP release to chemotaxis. Interestingly, in neutrophils the Pannexin-1 channel (PANX-1) determines the release of ATP and autocrine stimulation of the cell via P2Y2 and A_3 receptors [41]. Besides neutrophils, involvement of the P2Y2 receptor in chemotaxis has been demonstrated in eosinophils, macrophages and dendritic cells (DCs) [24,40,44]. Damaged neurons release UDP, which stimulates P2Y6-dependent chemotaxis of microglial cells; however, microglial cells lacking the P2Y12 subtype lose the capacity to polarize their plasma membrane, extend pseudopodia, and migrate towards brain-damaged areas in mice, suggesting that participation of this subtype is required in the 'preparatory' steps of chemotaxis [45]. Intriguingly, nucleotides are involved in chemotaxis stimulated by other chemoattractants, such as in the case of the

complement protein C5a. It has been elegantly shown that macrophages migrating in a C5a gradient release ATP that activates P2Y2 and P2Y12 receptors and, once degraded by ectonucleotidases, produces adenosine that stimulates P1 receptors in an autocrine manner [40]. Involvement of P1 receptors in chemotaxis of leukocytes has been shown in neutrophils, microglia, and eosinophils; however, the role of some P1 receptors (for example, the A_3 subtype) in chemotaxis remains controversial. While the A1 receptor promotes neutrophil chemotaxis [46] the A_{2A} and A_3 subtypes would inhibit it, and the A_{2B} receptor would be inhibitory for microglial chemotaxis [25,47–49]. Therefore, secretion of ATP and its hydrolysis to ADP, AMP, and adenosine are critical steps in the modulation of leukocyte chemotaxis.

What Is the Link between Chemokines and Purinergic Signaling?

Recent studies have highlighted the link between purinergic signaling and chemotactic pathways activated during the immune response, and compelling evidence shows that nucleotides and nucleosides are endowed with the ability to modulate chemokine secretion [50–53]. Cell chemotaxis is preceded by membrane polarization and important morphological changes paralleled by redistribution of intracellular signal transduction proteins implicated in motility, directional sensing, and polarity. These events include accumulation of the intracellular second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3), Rac-mediated actin polymerization, Rho and calcium modulation of actin- and myosin-mediated cell contraction, and redistribution of phosphatase and tensin homolog (PTEN) and myosin II [54]. Triggering of chemokine receptors by their ligands induces formation of intracellular phospholipids and calcium concentration increases [55]. Extracellular nucleotides seem to substantially contribute to chemokine receptor signaling by increasing the amplitude of the total calcium increase. Elegant experiments performed by heterologous expression of chemokine receptors in CHO cells indicate the need for P2Y-mediated pre-stimulation with ATP to induce CCR4-dependent Ca^{2+} concentration increase [56]. Accordingly, Ca^{2+} signaling induced by the binding of the chemokine **CXCL8** to the recombinant CXCR2 chemokine receptor is potentiated by pre-stimulation of cells with ATP or UTP and putative involvement of the P2Y1 and P2Y2 subtypes has been hypothesized [57]. Interestingly, a recent report showed that the monocytic cell line THP-1 rapidly secretes ATP on activation of the **monocyte chemotactic protein 1 (MCP-1)**–MCP1R axis and the authors suggest that P2Y6, which is not activated by ATP, might act as a coactivator of cell chemotaxis stimulated by CCL2 since P2Y6 activation is responsible for ~80% of the intracellular Ca^{2+} signal evoked by CCL2 in these cells [52]. Also peculiar is the fact that the same nucleotide has opposite effects on chemotaxis induced by two different cytokines. Thus, ATP inhibits natural killer (NK) cell chemotaxis in response to CX_3CL1 while it increases that stimulated by **CXCL12** [58]. In other cases nucleotides inhibit chemokine secretion; as an example, in human mast cells ADP and ATP block CXCL8 and CCL4 secretion in response to leukotriene D4 [59]. These findings indicate that nucleotides and nucleosides not only are chemoattractants *per se* [60–62], but: (i) induce/inhibit chemokine secretion; and (ii) modulate production of chemokines stimulated by proinflammatory mediators. In the following sections, we describe the role of extracellular nucleotides and nucleosides in modulating chemokine secretion and chemokine-mediated immune responses, highlighting the fact that P2 receptor stimulation promotes inflammatory responses by upregulating expression of various chemokine genes and inducing chemokine secretion. This may in some cases exacerbate inflammation, thus causing tissue damage. By contrast, P1 receptor activation in most cases inhibits chemokine secretion and reduces phagocyte recruitment, thus protecting tissues from immune-mediated cell damage.

P1 Receptors and Chemokines

Adenosine and its receptors play a fundamental role in the immune response mainly by down-modulating multiple cytokine expression and secretion [17]. Recent data have confirmed the role of adenosine in modulating chemokine secretion and chemokine receptor activation and a deep investigation has been undertaken to shed light on the complex interplay between adenosine

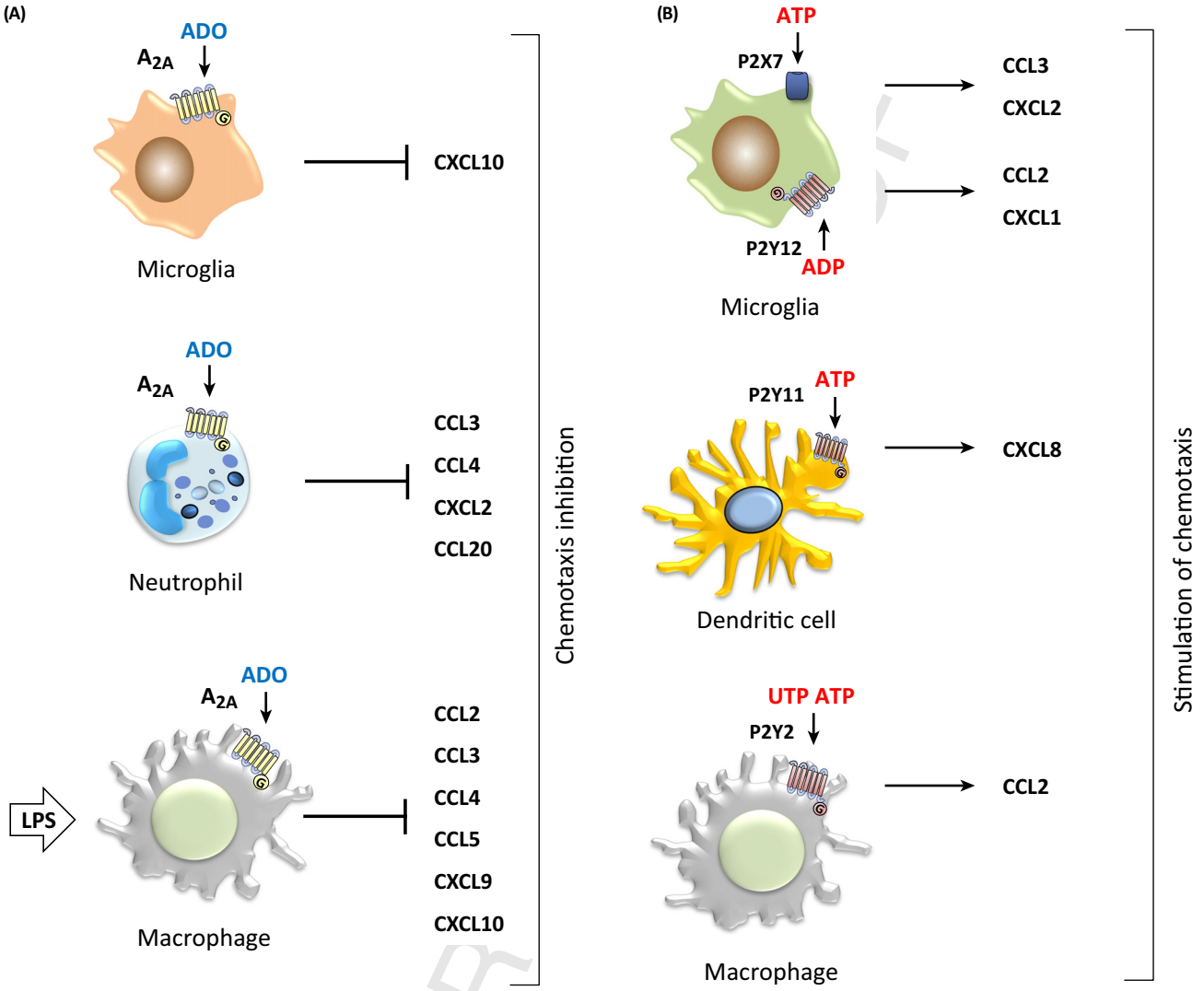

Figure 2. Purinergic Signaling and Chemokines. Involvement of specific P1 **(A)** and P2 **(B)** receptors in modulation of chemokine secretion. (A) Adenosine (ADO) reduces or inhibits secretion of various chemokines by immune cells. The A_{2A} subtype blocks CXCL10 production by microglia, CCL3, CCL4, CXCL2, and CCL20 by neutrophils, and CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 by macrophages stimulated with bacterial endotoxin (LPS). (B) ATP, UTP, and ADP stimulate chemokine secretion by activating different P2 subtypes. Microglia secrete CCL3 and CXCL2 on stimulation of the P2X7 subtype and CCL2 and CXCL1 as a consequence of P2Y12 triggering. Activation of the P2Y11 receptor induces CXCL8 secretion by dendritic cells, while the P2Y2 subtype is involved in CCL2 secretion by macrophages.

and chemokines in tuning leukocyte functions [63–67] (Figure 2A). Most of these studies indicate that adenosine through its receptors decreases chemokine production and dampens inflammation thus reducing tissue damage (Table 1). Hypoxic conditions induce adenosine formation in the extracellular milieu [10] and adenosinergic receptors are involved in hypoxia-related signaling pathways [68]. In particular, the A_{2A} subtype is involved not only in T cell apoptosis but also in the signaling pathway that reduces CCR7 expression under hypoxic conditions [69]. The same subtype participates in the downregulation of CXCR4 and CCR5 induced by an agonist-like monoclonal antibody [64]. A crucial aspect related to the establishment of hypoxic conditions is lack of infiltration of tumor mass by cytotoxic T lymphocytes. This is mainly due to stimulation by adenosine of the A_{2A} subtype [70]. The authors demonstrated that hypoxic conditions present in the tumor microenvironment (TME) were able to modify the cytokine and

Table 1. Nucleotides and Nucleosides Modulate Chemokine Secretion and Chemokine Receptor Expression

Receptor	Agonist	Effect on Chemokine/Chemokine Receptor		Species	Tissue	Refs
A_{2A}	ADO + Ab	CXCR4	↓	H	T cells	[64]
		CCR5	↓			
	2′,3′-cAMP	CXCL10	↓	M	Microglia	[67]
	3′-AMP					
	2′-AMP					
		CCR7	↓	H	T cells	[69]
		CCL3	↓	M	Neutrophils	[27]
		CCL4	↓			
		CXCL2	↓			
		CCL20	↓			
		CCL2	↓	H	Macrophages	[77]
		CCL3	↓			
		CCL4	↓			
	CGS 21680	CCL5	↓	H	Macrophages	[66]
		CXCL9	↓			
		CXCL10	↓			
		CCR7	↓			
A_{2B}	ADO	CCL-3	↓	M	Mast cells	[76]
A_3	IB-MECA	CCL3	↓	M	Macrophages	[72]
P2X7	ATP	CCL3	↑	M	Microglia	[50,85]
		CXCL2	↑			
	BzATP	CCL2	↑	M	Microglia	[29]
P2Y2	ATP	CXCL8	↑	H	Uroepithelial cells	[91]
	ATP/UTP	CCL2	↑	R	Macrophages	[92]
P2Y6	UDP	CXCL8	↑	H	Monocytes	[88,89]
P2Y11	NF546	CXCL8	↑	H	DCs	[93]
P2Y12		CCL2	↑	M	Microglia	[53]
		CXCL1	↑			

ADO, adenosine; Ab, antibody; H, human; M, mouse, R, rat.

chemokine repertoire towards immunosuppressive responses while elimination of adenosine and tissue reoxygenation reverted the antitumor immune response [71]. Adenosine has been implicated in regulating polymorphonuclear (PMN) cell transepithelial migration mediated by CXCR2 [65]. A_{2A} activation is involved in modulating the chemokine repertoire in human neutrophils by preventing the expression and release of MIP family molecules (MIP-1α/CCL3, MIP-1βa/CCL4, MIP-2α/CXCL2, and MIP-3α/CCL20), thus greatly reducing the responses mediated by these factors (i.e., chemotaxis, degranulation, phagocytosis, and production of inflammatory mediators that contribute to the onset and progression of inflammation) [27]. Stimulation of the A_3 receptor suppresses the production of CCL3 and exerts anti-inflammatory effects by decreasing neutrophil recruitment [72]; by contrast, adenosine increases CXCL8 secretion in monocytes/macrophages stimulated by IL-1β [73]. Moreover, the A_{2B} subtype evokes CXCL8 release in an ERK and p38 MAPK-dependent manner in human mast cells [74–76]. A recent report shows that the A_{2A} receptor inhibits LPS-induced

production of CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10 but not that of CXCL1, CXCL8, and CXCL5 in human lung macrophages [77] (Figure 1A). Monocytes migrate into tissues in response to various stimuli among which CCR7, and its chemoattractant agonist CCL21, plays a central role during the onset of inflammatory conditions underlying atherosclerosis. Interestingly, activation of the A_{2A} subtype modulates CCR7 expression under both normal and inflammatory conditions and tunes macrophage migration in response to CCR7-specific chemoattractants [66] (Table 1).

The role of chemokines in directing and activating immune cells has also been shown in the nervous system. The recent finding that mouse microglia possesses the extracellular 2',3'-cAMP–adenosine pathway converting released 2',3'-cAMP to adenosine prompted an investigation into the role of adenosine in modulating chemokine release by microglia; in particular, A_{2A} receptor stimulation exerts an inhibitory effect on CXCL10 production by activated primary murine microglia [67] (Figure 1A). A role for adenosine in driving migration of lymphocytes has also been described in the central nervous system. Hence, the nucleoside induces expression of CX3CL1 at the choroid plexus during experimental autoimmune encephalomyelitis (EAE) through immunization with myelin oligodendrocyte glycoprotein (MOG 35–55). It has been demonstrated that mice lacking expression of CD73 and consequently unable to produce extracellular adenosine do not upregulate CX3CL1 and do not show brain lymphocyte infiltration [78]. Moreover, the A_{2A} receptor subtype has been implicated as a mediator of this lymphocyte function, as inhibition of the subtype by the specific antagonist SCH58261 protects wild-type (WT) mice from EAE, which represents an experimental model for multiple sclerosis [79]. Another interesting finding is that the **fractalkine**–CX3CR1 signaling pathway plays a role in modulating microglial activation and neuron survival [80] and it is relevant that CX3CL1 is able to rescue neurons from neuronal excitotoxic death only in the presence of functional A_1 receptor, as confirmed by the fact that hippocampal neurons from A_1 receptor$^{-/-}$ mice are not rescued from Glu-induced cell death [81]. Therefore, modulation of adenosine receptors may be a strategy worthy of further evaluation for acute and chronic inflammatory disorders in the nervous and other systems.

P2X Receptors and Chemokines

Activation of the P2X1 receptor by ATP promotes neutrophil chemotaxis, a process involving Rho kinase-dependent actomyosin-mediated contraction at the cell rear [62]. Due to the massive recruitment of leukocytes, excessive chemokine secretion can be deleterious for tissue integrity [16,82]. P2 purinergic signaling is actively involved in the potentiation of chemokine secretion induced by leukocyte peptides such as the human neutrophil antimicrobial peptides, thus increasing expression of CXCL8 in the colonic mucosa of patients with active ulcerative colitis [30]. P2-mediated amplification of chemokine secretion also occurs in the case of bacterial toxin induction of CXCL8 secretion, which is the basis of intestinal epithelial barrier dysfunction [28]. The same is true for CCL2, which massively recruits monocytes in chronic inflammatory diseases and whose secretion consistently contributes to the inflammatory background of psoriasis, uveitis, multiple sclerosis, asthma, and chronic obstructive pulmonary disease [83]. A potentially interesting finding for new therapeutic approaches based on the modulation of chemokine secretion by purinergic signaling is the observation that the epidermis of psoriatic patients shows increased expression of P2X7 at the cell membrane of the basal layer [84]. Moreover, stimulation by ATP of IFN-γ-treated human keratinocytes induces a complex modulation of chemokine secretion with increased production of CCL2, CCL5, and CXCL8 and downregulation of CXCR3, CXCL9, CXCL10, and CXCL11 [84] (Figure 2B). P2X receptors have been shown to participate in human and mouse neutrophil chemotaxis induced by bacterial products such as formylated peptides or CXCL8 both *in vitro* and *in vivo*.

Microglia have the potential for both positive and negative effects in the central nervous system. Among signals able to 'overactivate' microglial cells and promote neurotoxicity, ATP has the

capacity to stimulate the release of inflammatory cytokines (IL-6, IL-1β, TNF-α) and chemokines [29,85]. Stimulation of the P2X7 receptor subtype by ATP or 2′(3′)-*O*-(4-benzoylbenzoyl)-ATP (BzATP) induces mRNA expression and release of the chemokine CCL2 in WT mouse microglia but not in $P2X7^{-/-}$ cells, showing that this subtype, and not other P2X receptors, is responsible for nucleotide-induced CCL2 production by microglial cells [29]. It has been shown that extracellular ATP induces activation of the transcription factor nuclear factor of activated T cells (NFAT) in mouse microglial cells and recent data indicate a role of NFAT in P2X7-induced CCL3 and CXCL2 secretion in microglia [50,85] (Figure 2B and Table 1). Peculiarly, stimulation of cells with chemokines also modulates nucleotide-mediated effects. Hence, pretreatment of microglial cells with CX_3CL1 significantly inhibits ATP-induced apoptosis in microglia and transforms, in a PI3-kinase-dependent manner, amoeboid microglia into quiescent, ramified forms [86]. Therefore, much effort is required to study the reciprocal effects of purines and chemokines with the aim of managing the undesirable effects caused by these systems.

P2Y Receptors and Chemokines

The involvement of Gi protein-coupled receptors, including P2Y receptors, in modulating chemokine secretion by immune cells emerged some years ago. This finding was supported by the observation that UDP stimulated the release of CXCL8 from LPS-matured human monocyte-derived dendritic cells, which are professional antigen-presenting cells obtained by *in vitro* differentiation of peripheral human monocytes in the presence of GM-CSF and IL-4 followed by maturation in the presence of bacterial endotoxin (LPS) [87]. LPS induces the release of ATP and UDP in monocytic cells thus promoting CXCL8 secretion in a P2Y6-mediated manner [88,89]. A requirement for this subtype for CXCL8 secretion has also been shown in nonimmune cells, as stimulation of lung epithelial cells with ATP or UDP induces CXCL8 secretion and inhibition of P2Y6 blocks it thus preventing recruitment of inflammatory cells (monocytes, neutrophils, etc.) to the lung parenchyma [90] (Table 1).

Bacterial toxins induce the release of chemokines attracting immune cells. *Clostridium difficile* toxins A and B, for example, trigger the release of CXCL8 from intestinal epithelial cells, attracting neutrophils that contribute to disruption of the intestinal epithelium. Intoxication of human epithelial colorectal adenocarcinoma (Caco-2) cells induces UDP release, P2Y6 activation, and increased CXCL8 expression [28]. Data obtained in mice with colitis-like disease indicate a role of the P2Y6 receptor in neutrophil recruitment to inflamed intestinal mucosa; the process is mainly due to increased CXCL8 expression evoking massive neutrophil recruitment [28]. Interestingly, enemas with the P2Y6 agonist UDP increase CXCL8 expression in mice and mutations at the AP-1 consensus site completely block the UDP-mediated effect [51]. Although P2Y6 is clearly involved in CXCL8 secretion, this subtype seems to be not essential for secretion of the cytokine in all systems. For example, neutrophil peptides induce secretion of the chemokine in the HT-29 cell line, which does not express the P2Y6 subtype [7,30]. The P2Y2 subtype is likely to be another candidate for purinergic-mediated CXCL8 secretion since stimulation of uroepithelial cells by ATP induces P2Y2-mediated release of CXCL8 [91]. Moreover, in rat peritoneal and alveolar macrophages (NR8383 cell line), constitutive CCL2 release is increased by ATP or UTP via the P2Y2 subtype [92]. While extracellular ATP protects endothelial cell from CX_3CL1-stimulated NK killing activity in a P2Y11-dependent manner [58], the same subtype stimulates CXCL8 secretion by Ds [93]. The P2Y12 subtype has recently been shown to modulate CCL2 and CXCL1 release by mouse microglia in brain tissue slices and blockade of this subtype inhibits CCL2 and CXCL1 secretion [53]. Different chemokines are involved in the pathological recruitment of lymphoid cells, a characteristic feature of several chronic inflammatory diseases. Among them, CCL2 plays a role in recruitment of monocytes in psoriasis, rheumatoid arthritis, and atherosclerosis [94]. Due to the fact that different P2Y subtypes (P2Y2, P2Y6, P2Y12) stimulate the secretion of chemokines involved in pathological states, clarification of the interplay between purinergic P2Y-mediated signaling and chemokine

secretion would contribute to improving our knowledge of the pathogenesis and progression of these diseases and is likely reveal novel therapeutic targets.

Ectonucleotidases and Chemokines

Nucleotide-metabolizing enzymes play a role in controlling the concentration of nucleotides available for P1 and P2 receptor activation [12,13]. In particular, their activity decreases ATP and ADP concentrations, thus dampening P2 receptor-mediated responses, while increasing adenosine concentration and, consequently, 'protective' P1-induced effects. Recent data indicate a role for these enzymes in modulating the secretion of various chemokines, and pharmacological inhibition or lack of expression of these enzymes has the net effect of increasing chemokine production. Human neutrophils express NTPDase1 (CD39) and inhibition of this enzyme greatly increases CXCL8 secretion in TLR-stimulated human neutrophils. Accordingly, injection of LPS in the air pouches of NTPDase1-deficient mice leads to increased secretion of the rodent counterparts of human CXCL8, the chemokine CXCL2 and keratinocyte-derived chemokine [95]. Another recent report showed that degradation of extracellular nucleotides with apyrase greatly decreases THP-1 sensitivity to CCL2, while inhibition of CD39-like ectonucleotidases potentiates CCL2-induced Ca^{2+} responses [52]. Although further investigation is needed to confirm and integrate initial observations on the interplay between ectonucleotidase activity and chemokine secretion, we can hypothesize that ectonucleotidases have the ability to dampen inflammation by increasing adenosine concentration in the extracellular compartment and consequently decreasing chemokine production and excessive immune cell recruitment.

Concluding Remarks and Future Perspectives

Exaggerated inflammatory response is the basis of the pathogenesis of various diseases such as psoriasis, rheumatoid arthritis, atherosclerosis, inflammatory bowel disease, asthma, and multiple sclerosis [97]. Therefore, it is urgent to identify mechanisms and pathways underlying pathologic inflammatory states to reveal new therapeutic targets and novel treatments (see Outstanding Questions). Although chemokines are needed to mount an adequate defensive response, excessive chemokine secretion induces abnormally high leukocyte recruitment paralleled by increased release of toxic mediators with consequent tissue damage. Dysregulation of chemokine expression causes excessive recruitment of leukocytes [98,99]. Various attempts are ongoing to therapeutically target chemokines by specific inhibitors and to find novel anti-inflammatory and proresolutive molecules [83]. Purinergic signaling has been the focus of increasing attention with the aim of dampening P2 proinflammatory and amplifying P1 anti-inflammatory responses [99,100]. Since P1 receptors down-modulate and P2 receptors up-modulate chemokine expression and secretion, in future trials it will be important to pay more attention to the interplay between purinergic signaling and chemokines to reduce/control the negative effects of excessive chemokine production in diseases in which abnormal recruitment of immune cells and subsequent repeated induced tissue damage are critical pathogenic factors, such as autoimmune and chronic inflammatory diseases.

Q2 **Uncited references**

[35,37,42,43,96].

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Outstanding Questions

What is the potential for blocking the release of chemokines involved in chronic inflammatory diseases such as atopic dermatitis and allergic asthma by targeting purinergic signaling?

Is excessive chemokine release associated with changes in extracellular ectonucleotidase expression? Could basic and translational studies be used to determine whether downregulation of ectonucleotidase expression or activity could reduce degradation of the proinflammatory P2 agonist ATP? How might this effect the formation of the anti-inflammatory P1 agonist and the establishment of a proinflammatory background?

How can purinergic signaling pathways be targeted to treat patients who are experiencing acute or chronic inflammatory diseases? Do inhibitors of specific P2 purinergic receptors currently used in clinical trials inhibit excessive production and/or secretion of pathogenically involved chemokines?

How does nucleotide and nucleoside release regulate the outcome of inflammation through chemokine secretion? As nucleotides and nucleosides exist in the intracellular and the extracellular compartment, what is their relationship and function to alter immunologic responses? Since there are many pathways that control nucleotide and nucleoside efflux across the cell membrane, is excessive chemokine secretion supported by increased efflux of ATP through the different families of deputed molecules (connexines, pannexines, membrane transporters)?

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