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Review article

Tinkering with targeting nucleotide signaling for control of intracellular *Leishmania* parasites

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ABSTRACT

Nucleotides are one of the most primitive extracellular signalling molecules across all phyla and regulate a multitude of responses. The biological effects of extracellular nucleotides/sides are mediated via the specific purinergic receptors present on the cell surface. In mammalian system, adenine nucleotides are the predominant nucleotides found in the extracellular milieu and mediate a constellation of physiological functions. In the context of host-pathogen interaction, extracellular ATP is recognized as a danger signal and potentiates the release of pro-inflammatory mediators from activated immune cells, on the other hand, its breakdown product adenosine exerts potential anti-inflammatory and immunosuppressive actions. Therefore, it is increasingly apparent that the interplay between extracellular ATP/adenosine ratios has a significant role in coordinating the regulation of the immune system in health and diseases. Several pathogens express ectonucleotidases on their surface and exploit the purinergic signalling as one of the mechanisms to modulate the host immune response. *Leishmania* pathogens are one of the most successful intracellular pathogens which survive within host macrophages and manipulate protective Th1 response into disease promoting Th2 response. In this review, we discuss the regulation of extracellular ATP and adenosine levels, the role of ATP/adenosine counter signalling in regulating the inflammation and immune responses during infection and how *Leishmania* parasites exploit the purinergic signalling to manipulate host response. We also discuss the challenges and opportunities in targeting purinergic signalling and the future prospects.

1. Introduction

Purines nucleotides are the most primitive and ubiquitous signaling molecules across all phyla [1]. Purine and pyrimidine nucleotide/side are largely confined to intracellular space where they are involved in the intracellular energy transfer and synthesis of genetic material [2,3]. However, they can also be released into extracellular space under myriad physiological conditions such as hypoxia, tissue injury, infection etc. and so releases nucleotides act as autocrine or paracrine signalling molecules by occupying their distinct receptors on the cell surface [4]. The purine and pyrimidine receptors are collectively termed as purinergic receptors and are broadly classified into two types; P1 adenosine receptors and P2 nucleotide receptors, based on their affinity for adenosine and other nucleotides (mainly ATP) respectively [5,6]. The extracellular nucleotides concentration and thereby the outcome of the purinergic signalling is controlled by the ectonucleotidases present on the cell surface or in the extracellular milieu [7,8].

ATP is one of the most critical biological molecules; it participates in more chemical reaction than any other molecules (except water) in the biological system. Extracellular ATP (eATP) is sensed at all phylogenetic level and the basic structure and functions of the P2X receptor (P2XR) remain conserved during evolution (Table 1) [9–12]. ATP is omnipresent in all organisms and is released in response to various environmental stimuli; therefore, it is believed to be evolved as an extracellular signaling molecule early in the evolution. Looking at the modus operandi of the evolution of early life forms, it is reasonable to conjecture that small diffusible molecules such as amino acids (γ-aminobutyric acid), a gaseous transmitter (nitric oxide) or other small molecules could essentially be served as cell-to-cell communicating molecules among the early ocean life forms [13]. Indeed, diffusion of ATP is the most ancient and general mechanism found throughout phylogeny. Thus, ATP possesses all the traits of a bonafide fast-acting intracellular and extracellular signaling molecule: (a) owing to its small size and high mobility, ATP is released in response to external stimuli in

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Table 1
List of evolutionarily conserved P2X receptors in some of the selected organisms.

Organism	Receptor	% Similarity with human	Sensitivity	Reference
Lymanae stagnales (pond snail)	$LymP2X_7$	30–46%	ATP, BzATP, α,β- anerylene ATP, PPAD, Suramin	[9]
Boophilus Microplus (tick)	Bm P2X	30–40%	ATP, Suramin	[10]
Dictyostelium discoideum	$DdP2X_{A-E}$	23 – 38%	ATP	[14]
Danio rerio (Zebrafish)	zP2X	45–55%	ATP, ADP	[11]
Hypsibius dujuardini	$HdP2X_7$	36–38%	ATP, BzATP, α,β- anerylene ATP. PPAD, Suramin	[12]
Ostreococcus tauri	OtP2X	28%	ATP, BzATP, α,β- anerylene	[14]
Schistosoma mansoni	Sch P2X	35–37%	ATP, BzATP, PPAD, Suramin	[274,275]
Typanosoma cruzi	2 P2X	?	ATP	[275]
Monosiga brevicolis (Choanoflates)	MbP2X	?	ATP, ADP	[14]

a controlled fashion, (b) acts on distinct membrane receptors and (c) is quickly cleared from the system to terminate its action. Ubiquitous distribution of several purinergic receptor and ectonucleotidases virtually on all organisms substantiates this conjecture [1]. The significant homology of P2XR between higher eukaryotes and lower vertebrates and invertebrates suggests that P2XRs are evolutionarily conserved (Table 1). This is in accordance with the view "purinergic signaling may be among the oldest signaling mechanism" [14,15].

The eATP is a bonafide ligand to P2X receptor across phyla and assumes a diverse role in different organisms ranging from chemotaxis to cell differentiation and apoptosis (Table 2) [16–21]. Both host and pathogen have evolved to sense the extracellular nucleotides and mount an appropriate response in a race to adapt to the presence of one another [22]. In the mammalian immune system, eATP always sensed as a danger signal and triggers an inflammatory response [23]. Conversely, its breakdown product, adenosine exerts either immunostimulatory or immunosuppressive function depending on the concentration it achieved and the type of receptor it activates [3]. Several, if not all, pathogens equipped with the surface as well as secretory ectonucleotidases which sequentially degrade eATP to adenosine at the site of infection (Table 3) [24–26]. In addition, hematophagous insects and ticks inject ectonucleotidases, adenosine monophosphate (AMP) and adenosine at the site of their bite to minimize the ensuing pain and activation of local immune system [27]. It is interesting to note that, adenosine transporters are found in both host and pathogen, however till date adenosine receptors have been reported in higher eukaryotes but not in pathogens, it augurs well with the conjuncture that the adenosine generated during host-pathogen interaction not only restrict to the immuno-suppressive response but also be used for parasite growth development [28]. Therefore, the ATP/adenosine axis is posited as a powerful evolutionarily selected mechanism deputed for the fine-tuning of inflammatory responses and tissue protection. The co-expression of multiple purinergic receptors and ectonucleotidases on immune cells and selective up-regulation of these receptors and ectonucleotidases on the cell surface during inflammatory response underpins the role of ATP/adenosine in the regulation of inflammatory homeostasis [29].

Purine and pyrimidine nucleotides are implicated in a large of physiological and pathophysiological functions in mammalian cardiovascular, nervous system as well as in immunological defense mechanisms. The purpose of this review is not to collate citation for a comprehensive review; but to focus on how extracellular ATP and adenosine reciprocally regulate the mammalian immune system and how pathogens exploit purinergic signalling for disease progression, emphasis on its importance in the modulation of host immune system during *Leishmania* infection.

2. P2 purinergic receptors diversity and their functions

In all life forms, energy is stored in the form of ATP molecule, therefore, it is considered as "universal energy molecule" responsible for intracellular energy transfer. Until 1970, no one envisioned the role for the ATP outside the cell. It was ascribed that, the presence of nucleotides/sides in the extracellular milieu was the result of cell death or tissue injuries, and no biological significance was assigned to it. In 1970, Burnstock discovered ATP as non-adrenergic and non-cholinergic neurotransmitter (NANC) [30], nonetheless large part of the scientific community remained skeptical about ATP as an extracellular signaling molecule because the existence of ATP receptor was not demonstrated until1978. Cloning of multiple nucleotide receptors in early1990s opened the door for immunohistochemical studies and revealed that most neuronal, as well as non-neuronal cells, express multiple P1 and P2 receptors [31,32]. Generally, ATP is confined to intracellular compartments where it serves as energy currency for intracellular metabolic activities. The intracellular ATP concentration is maintained approximately at 5–10 mM, whereas owing to the ectonucleotidase activity, extracellular ATP concentration does not exceed 1–10 nM. Due to this difference in the ATP gradient (a difference of approximately 1 million times) and owing to its small size and high mobility, cells constitutively release a basal level ATP into the extracellular space through electro-

Table 2
Functions of ATP in some of the selected organisms.

Organism	Nucleotide/nucleoside	Function	References
Dasycladus vermicularis	ATP	Wound response	[16]
Acetabularia acetabulum	ATP	Wound response	[16]
Leishmania	ATP	Chemotaxis	[22]
Molluscs	ATP	Immune cell motility	[17]
Dictyostelium discoideum	ATP	Osmo regulation	[14]
Tetrahymena thermophila	ATP	Chemotaxis (swim away, avoiding response)	[18]
Paramecium	ATP and GTP	Chemotaxis (swim away, avoiding response)	[19]
Schistosoma mansoni	ATP	Opening of cationic gates and pore formation in plasma membrane	[274]
Actinia equina	ATP and ADP	Hair bundle repair	[20]
Bacteria	ATP	Adhesion and biofilm formation	[21]
Animals	ATP, ADP, Adenosine, UTP and UDP	Immunehomestasis, neural signalling and embryonic development	[37,78]
Higher plants	ATP	Sensing presence of pathogen, Production of NO and ROS	[36]

Table 3
Ectonucleotidases involved in the virulence of selected pathogens.

Pathogen	Enzyme involved	Action	References
Trichinella spiralis	Secretory 5′-nucleotidase, NDP	Inhibit the release of protease 1. Invasion of mucosal epithelium	[24]
Mycobacterium bovis	NDPK and ATPase	Prevention of ATP mediated apoptosis	[25]
Pseudomonas aeruginosa	NDPK, ATPase, Adenylate kinase, ATPase	Prevention of ATP mediated apoptosis. Generation of adenosine	[26]
Candida parapsilosis	5-ectonucleotidase	Adhesion to host macrophages and internalization	[137]
Staphylococcus aureus	Adenosine synthase A	Escapes from phagocytosis	[138]
Streptococcus suis	Adenosine synthase	Escapes from phagocytosis	[141]
Streptococcus sanguinis	5-ectonucleotidase	Inhibits platelet aggregation, increase bacterial vegetation Survival inside the host blood	[139]
Porphyromonas gingivalis	NDK	Colonization of parasites in oral tissues. Inhibits ATP mediated apoptosis and parasite clearance.	[143]
Trypanosoma cruzi	E-NTPDase	Increased production of adenosine Promote infection	[211]
Leishmania amazonensis	NDK	Inhibits apoptosis of macrophages	[212]
	E-NTPDase	Supresses nitric oxide production from macrophages	
Leishmania amazonensis	3′-ectonucleotidase	Involve in host pathogen interaction	[212]
Leishmania infantum	3′-Nucleotidase/Nuclease	Escape killing by Neutrophil Extracellular Traps	[221]
Taxoplasma gondi	NTPase I and II	Increases Infection	[101]

NDPK = Nucleotide diphosphate kinase.
NDPase = Nucleotide diphosphatase.
NTPase = Nucleotide triphosphatase.
E-NTPase = Ecto- nucleoside triphosphate diphosphohydrolase.

diffusion or facilitated diffusion [33–35]. This low concentration of eATP that exists in a “halo” surrounding quiescent cells signals the presence of neighboring living cells. However, a steep increase in the eATP due to cell death or injury or exocytosis of ATP in response to external stimuli serves as “danger signal” and elicits the inflammatory response [33]. Hence, eATP is considered as the prototypical danger associated molecular pattern (DAMP) and is recognized by membrane-bound pattern recognition receptors (PRRs) *via* P2 purinergic receptors [23,36]. Virtually all the cells in the body can release a variety of nucleotides [37], among these, adenine nucleotides (ATP, ADP, AMP and adenosine) are the predominant nucleotides released into the bloodstream [38,39]. Although ischemia, anoxia, microbial invasion can induce ATP release, there are multiple physiological stimuli that can also stimulate the release of ATP from different cells and tissues. Nucleotides can release into extracellular milieu either by specifically through equilibrative nucleoside transporters (ENTs) and the concentrative nucleoside transporters (CNTs) or non-specifically through connexin and pannexin hemichannels or exocytosis Fig. 1 [40,41]. This eATP is a potent endogenous ligand for many P2 purinergic receptors and ligation of ATP to individual receptor elicit different responses (Table 4).

Ligation of ATP to P2 receptors activate and sustain an array of immunological responses. However, over-exuberant activation of the immune system can inflict unacceptable levels of collateral tissue damage and the development of various pathophysiological conditions, such as allergy, arthritis, inflammatory bowel disease etc. [42,43]. Therefore, termination of eATP induced inflammation is imperative to minimize the collateral tissue damage. Termination of eATP signalling is mediated by four families of ectonucleotidases *viz* nucleotide pyrophosphatases/phosphodiesterases (NPPs), nucleoside triphosphate diphosphohydrolases (NTPDases), alkaline and acid phosphatases and ecto-5′-nucleotidase [7,44–46]. Of these, NTPDasees (CD39) and ecto-5′-nucleotidase (CD73) abundantly expressing on the hematopoietic cells, especially on T regulatory (Treg) cells and dramatically control the extracellular ATP and adenosine concentrations [47–49]. The magnitude and duration of eATP and its metabolite depends on the bioavailability of nucleotide present in the extracellular milieu, the half-life of the nucleotide, and the type of purinergic receptors expressing on the proximate cells.

Currently, seven ionotropic ATP-gated P2X receptors (P2X1-7) and eight metabotropic G-protein coupled P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors have been cloned and pharmacologically characterized in mammals [50–57]. P2XRs in vertebrates share 40–50% identity in amino acid sequence and show similar pharmacological properties [58]. On the other hand, P2YRs share 19–55% identity at the peptide level, and as a result, individual members of the P2YRs exhibit a marked difference in pharmacological properties [29]. While P2XRs subtypes are activated by ATP, P2YRs show complex ligand binding properties (Table 4) [59]. Among P2XRs, the P2X7R subtype is a unique receptor, it has extended cytoplasmic tail, is activated at higher ATP concentrations and induces structural changes in the cell such as macropore formation in the membrane, induction of apoptosis, activation of MAP kinase and ultimately cell death [60,61]. Therefore, P2X7R is critically required for the lysis of the infected host cell and elimination of intracellular pathogens. Asides P2XRs, P2YRs are also functionally active during nuromodulation, chemotaxis, platelet physiology and other ancillary pathways (Table 5) [62–69]. P2X7R and $P2Y_2R$ subtype receptors are the most extensively characterized P2 receptors and both are activated by eATP and its analogs [70]. Since ATP is equipotent to P2X7R and $P2Y_2R$, the protective role of the eATP presumed to be mediated via P2X7R and $P2Y_2R$ cross-talking [21]. In the systemic inflammation model, removal of eATP found to be superior over blocking of P2X7R for controlling inflammation [71]. This suggests that eATP plausibly exerts its effects via activation of more than one P2 receptors [72,73]. The plasma membrane macropore formation is mediated through P2X7R activation [74], whereas, other functions such as recruitment of effector cells, maturation of dendritic cells and production of nitric oxide (NO) may be mediated by other P2 receptors [68,75–78]. Therefore, the antimicrobial activity of eATP is likely to be mediated by synergistic activation and cross-talks of multiple P2 purinergic receptors [79,80].

3. P1 purinergic receptors and their functions

A seminal work by Drury and Szent-Gyo¨rgyi in 1929 established the role of adenosine as an extracellular signaling molecule [81]. Later, in 1934, Gillespie demonstrated that the removal of an amine group or a phosphate group from adenine compounds not only alter their pharmacological potency but also the responses in mammalian cells [82]. Although the role of adenosine was established long ago, the detailed study of adenosine function was revealed in recent past due to the cloning and characterization of adenosine receptors from several mammalian species [32,83]. There is a strong evidence that adenosine

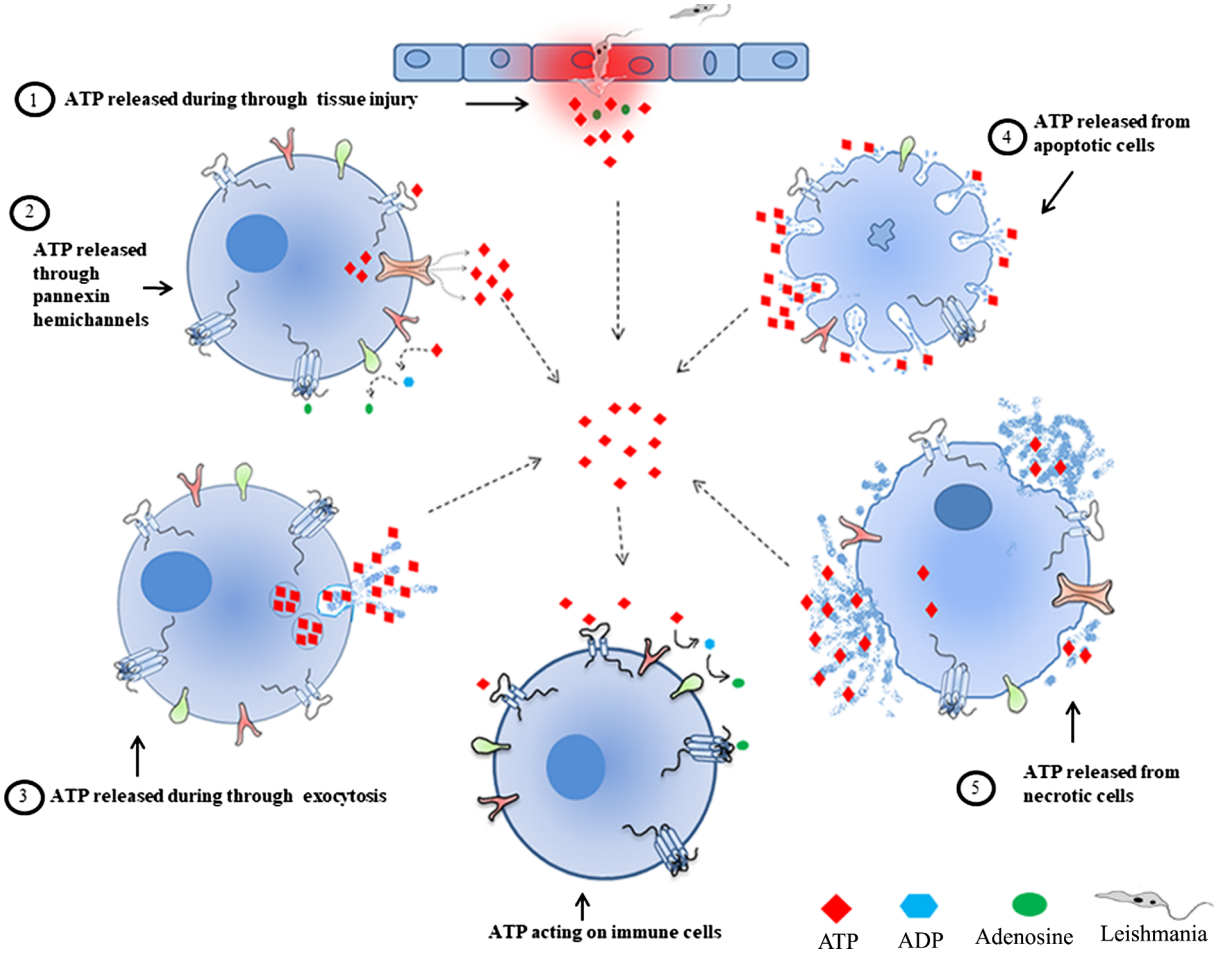

Fig. 1. Sources of extracellular ATP: ATP is the most abundant nucleotide found in the extracellular milieu. The extracellular ATP in the body is released by various activities such as (1) ATP released during tissue injury or (2) ATP secretion through pannexin hemichannels or (3) ATP released from cells through exocytosis or (4) ATP released from apoptotic cells or (5) ATP released from necrotic cells. So released ATP activate nearby immune cells through P2X receptors or converted into adenosine by surface ectonucleotidases.

Table 4
Purinergic receptors, their endogenous ligands and mode of action.

Receptor	Preferred natural ligands	Coupling	Mode of action	Reference
$P2X_1$	ATP		Ca2+ and Na+ influx	[50]
$P2X_2$	ATP		Ca2+ influx	[50]
$P2X_3$	ATP		Cation influx	[50]
$P2X_4$	ATP		Ca2+ influx	[50]
$P2X_5$	ATP		Ion influx	[51]
$P2X_6$	ATP		Ion influx	[52]
$P2X_7$	ATP		Cation influx and pore formation	[72]
$P2Y_1$	ADP > ATP	Gq	Activate phospholipase C-β,	[53,54]
$P2Y_2$	UTP = ATP > UDP	Gq	Activate phospholipase C-β,	[53,54]
$P2Y_4$	UTP > > ATP	Gq	Activate phospholipase C-β,	[55]
$P2Y_6$	UDP > UTP > ADP	Gq	Activate phospholipase C-β,	[53,54]
$P2Y_{11}$	ATP > ADP	Gq, Gi	Activate phospholipase C-β, Inhibit adenylyl cyclase	[56]
$P2Y_{12}$	ADP > ATP	Gi	Inhibit adenylyl cyclase	[56]
$P2Y_{13}$	ADP > ATP	Gi	Inhibit adenylyl cyclase	[56]
$P2Y_{14}$	UDP Glucose > UDP Galactose	Gi	Inhibit adenylyl cyclase	[57]
A_1	Adenosine High affinity	Gi,Go	Inhibit adenylyl cyclase Activate phospholipase C-β, PKC	[90]
A_{2A}	Adenosine High affinity	Gs, Golf	Increase adenylate cyclase	[91]
A_{2B}	Adenosine low affinity	Gs, Gq	Increase adenylate cyclase	[91]
A3	Adenosine High affinity	Gi,Go	Inhibit adenylyl cyclase Activate phospholipase C-β,	[92]

Table 5
Physiological and immunological functions of purinergic receptors.

Receptor	Function	References
P2X1	Release of IL-8 secretion in neurons	[50]
P2X2	Sensory regulation of visceral function	[244]
P2X3	Regulate acute and chronic pain Regulate bladder distension	[244]
P2X4	Chemotaxis of microglial cells and T cells Macrophages and T cell activation Neuropathic pain	[95] [71]
P2X5	Regulation of embryonic muscle development	[95]
P2X6	Regulation of embryonic muscle development	[95]
P2X7	Th1 and Th17 cytokine production Maturation of dendritic cells. Secretion of IL-8, IL-1β and IL-18 from various immune cells	[48,151]
P2Y1	Platelet aggregation, vasodilation.	[62]
P2Y2	TLR induced neutrophil migration by regulating IL-8 secretion, ROS production and prostaglandin E2 (PGE2) Monocyte chemo attractant (MCP-1) Regulation of ion transport in epithelial tissues	[63–65]
P2Y4	Modulation of retinal function	[66]
P2Y6	Promote IL-12 and IL-8 secretion. Promote TLR induced neutrophil migration. Regulation of ion transport in epithelial tissues	[64,67]
P2Y11	Maturation of dendritic cells, production of proinflammatory cytokines	[68]
P2Y12	ATP induced chemotaxis Platelet aggregation	[272]
A1	Promote Neutrophil chemotaxis and phagocytosis Promote production of IFN-γ and IL-2 from T cells	[104]
A2A	Inhibits the production of IL-12, TNF-α and IFN-γ Inhibit neutrophils chemotaxis and ROS production Promote TGF-β, IL-10 and IL-4 production	[67,106,107]
A2B	Promote persistent microbial infection Promote IL-10 and IL-6, decrease IL-12 and NO Down-regulate MHC II, CD40, CD80 and CD86 on DC Down-regulate ICAM and E-selectin expression	[101,112]
A3	Promote histamine from mast cell Promote neutrophil chemotaxis	[76,110]

has a role in physiological processes and can be used as a therapeutic drug molecule. Generic drugs such as Adenocard and Adeoscan which contain pure adenosine in physiological saline are directly used for clinical treatment of cardiac dysfunctioning [84,85], and many other clinically important drugs such as methotrexate and dipyridamole act by increasing the local concentration of adenosine [86,87]. Adenosine is either generated intracellularly by the enzymatic hydrolysis of S-Adenosyl-L-Homocysteine (SAH) by SAH hydrolase and refluxed into extracellular milieu (during hypoxia) via adenosine transporters or formed in the extracellular space owing to sequential degradation of extracellular ATP by CD39-CD73 enzyme cascade [88,89]. In cardiovascular tissues, during hypoxia extracellular adenosine is increased due to efflux of intracellularly formed adenosine, whereas in immunological context, extracellular adenosine is formed from the sequential degradation of ATP to adenosine by surface ectonucleotidases present on the surface of immune cells.

Extracellular adenosine mediates its biological effects via distinct adenosine receptors present on the cell surface. Four subtypes of adenosine receptors *viz* A_1, A_{2A}, A_{2B} and A_3 are cloned and characterized [90–92]. The A_1 receptor (A_1R) is the most widely distributed receptor among receptor subtypes, with the higher expression found within CNS and mediate diverse biological effects [93,94]. A_{2A} receptor ($A_{2A}R$) also has a wide distribution that includes immune tissues, visceral organs, blood vessels and platelets [95]. A_{2B} receptor ($A_{2B}R$) present practically on every cell, but mostly in low abundance. However, its expression steeply increases during pathophysiological condition and requires a high concentration of adenosine to evoke a response [96]. A_3 receptor (A_3R) is the most variable receptor among adenosine receptor subtypes and exhibits the lowest degree of identity among species [32,97], its physiological role is not fully resolved. Adenosine receptors share approximately 50% amino acid homology within receptor subtypes and > 80% homology is observed within species orthologs [98–100].

Adenosine receptors are G-protein coupled receptors, principally act via stimulation (Gs) or inhibition (Gi) of adenylatecyclase. A_1R and A_3R act via inhibition of intracellular cyclic-AMP (cAMP), whereas $A_{2A}R$ and $A_{2B}R$ increase intracellular cAMP production via Gs (Table 4) [101]. The physiological concentration of adenosine is critical for the homeostasis of the immune system. At low physiological concentrations, adenosine activates A_1R which acts as immunostimulant [102–104]. Whereas, during pathophysiological conditions or at inflammatory tissue microenvironment, the extracellular adenosine concentration increases with a parallel increased expression of A_2Rs [105]. A_2Rs are predominantly expressed by macrophages, neutrophils and dendritic cells, at a low level by lymphocytes and platelets [106]. A2 adenosine receptors have been recognized as the major deactivators of macrophage functions, inhibit the production of pro-inflammatory cytokines, down-regulates MHC class II expression and also leads to the switching of M1 to M2 macrophage polarization [3,107,108]. Apart from being scavenger and antigen presenting cells, macrophages are also the definitive hosts for many intracellular pathogens. Thus, deactivation of the macrophages not only hampers the phagocytosis and antigen presentation, but also activation of T cells via T cell receptor (TCR) signalling and recruitment of neutrophils through chemokines [3]. Besides tissue resident macrophages, inflammatory macrophages and neutrophils are also recruited to the infection site and serve both as host and effector [109]. Adenosine blunts the chemotaxis, degranulation and reactive oxygen species (ROS) production in neutrophils via activation of $A_{2A}R$ and $A_{2B}R$ [107,110]. In addition to macrophages and neutrophils, dendritic cells (DCs) are also the essential players of the immune system which serve as the bridge between the innate and adaptive arms of the immune system. Matured DCs produce an array of cytokines mainly IL-12 which is critically required for the differentiation of IFN-γ-producing Th1 lymphocytes involved in the elimination of infected host cells [111]. Adenosine severely impairs the maturation and antigen presenting functioning of dendritic cells. “Adenosine differentiated dendritic cells” exhibit tolerant phenotype properties which secrete lower IL-12 and higher IL-10 level, consequently impaired ability to promote IFN-γ producing Th1 lymphocytes [112–114]. Similarly, adenosine suppresses the T cell activation, survival, expansion and memory cell formation (effect of adenosine on T cells is discussed in detail in the next section) [115–117]. Therefore, adenosine suppresses effector function and dampens the cognate cross-talk between the various immune cells.

The local concentration of adenosine and adenosine receptors are extremely important for its immuno-suppressive functions [8,89,96]. A low level of adenosine (1 μM) is constitutively present in the extracellular milieu in the resting tissues [35,118]. During pathophysiological condition, the adenosine concentration can increase up to 100–150 folds [119]. Upon its release into the extracellular compartment, adenosine can activate one of the four adenosine receptors, depending on the concentration achieved in the extracellular microenvironment. Among all four adenosine receptors, the $A_{2B}R$ has the lowest affinity for adenosine [120]. Therefore, it is presumed that the $A_{2B}R$ is quiescent under the physiological conditions when extracellular adenosine concentration is low (0.2–0.5 μM), but its role becomes pivotal in pathophysiological condition when extracellular adenosine concentration becomes high (16.2–64.1 μM) [120]. Therefore, the $A_{2B}R$ is believed to play important role in the immune-suppression during chronic pathophysiological conditions. A_1R has a high affinity for adenosine and required for the normal physiological and cardiovascular functioning. Similarly, $A_{2A}R$ has a high affinity for adenosine, however under normal physiological condition its expression is very low; hence, its immuno-suppressive effects are masked, however, under acute inflammatory settings $A_{2A}R$ level is up-regulated and mediates the suppression of inflammation. When A_1R and A_2R co-expressed on the same

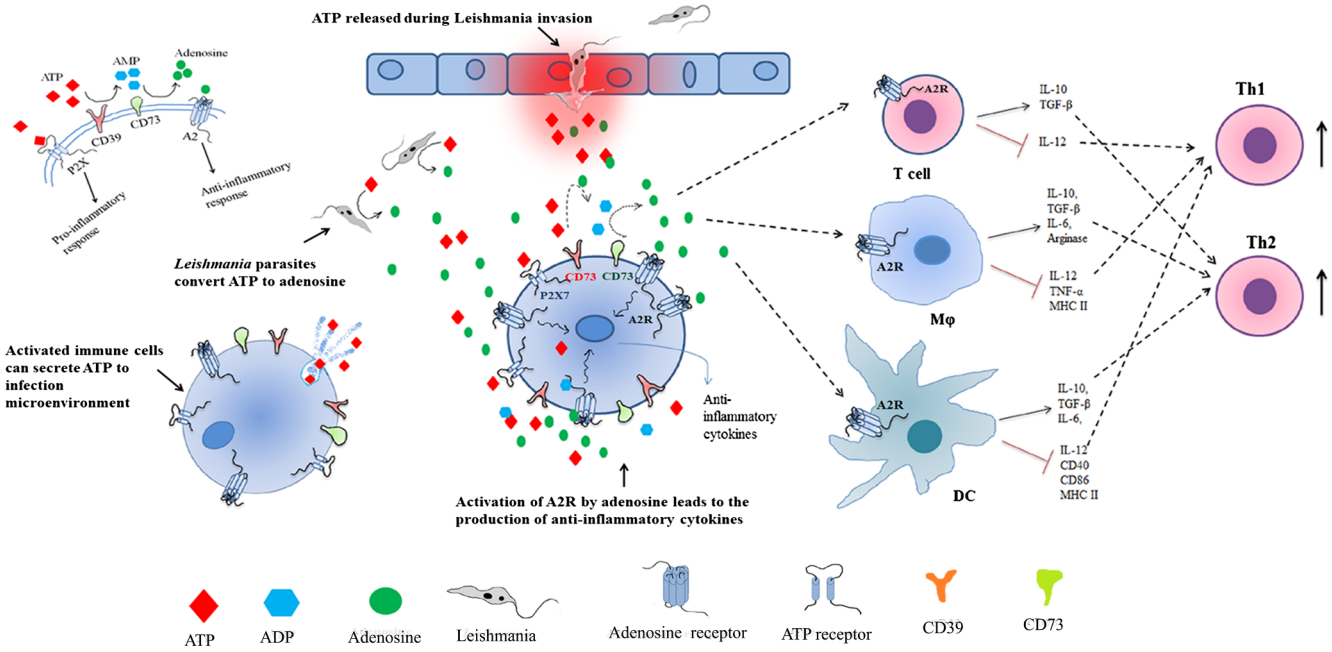

Fig.2. Adenosine-mediated immuno-modulation during leishmaniasis: ATP released during *Leishmania* invasion or due to mechanical damage resulting from an attack by blood feeding sandfly or the activated immune cell (by exocytosis) is degraded into adenosine by surface ectonucleotidases present on the host as well as parasite's surface. The adenosine generated by ectonucleotidase binds to A_2Rs (A_{2A}R and A_{2B}R) present on various immune cells which promote the secretion of Th2 cytokines and suppress the expression of co-stimulatory molecules on antigen presenting cells.

cell, activation of A_{2A}R inhibits the affinity of adenosine for A_1R, but not vice versa [121]. During acute inflammatory condition, activation of A_{2A}R inhibits the simultaneous activation of A_1R and suppresses the production of pro-inflammatory cytokines. However, during chronic inflammatory condition, where extracellular adenosine concentration reaches its peak leads to activation of low-affinity A_{2B}R promotes the alternative activation of macrophages which are critically required for the tissue repair [108]. Thus, the presence of four adenosine receptor subtypes with different affinity for endogenous adenosine and event specific expression of adenosine receptor underlines the relevance of different subtypes of adenosine receptors in the regulation of immune system.

4. ATP/adenosine axis in the regulation of pathogenesis

The immune system is broadly classified into two types; humoral immunity and cell-mediated immunity. Humoral arm primarily deals with antibody-mediated neutralization of pathogen and their products. Cell-mediated immunity consists of a heterogeneous population of immune cells which are involved in the clearance of dead and apoptotic cells, capturing and destruction of pathogens and elimination of infected cells [122]. T lymphocytes play a key role in the homeostasis of the immune system which is orchestrated through the production of various cytokines and chemokines. In addition, several soluble factors released from dead and injured cells can play major role in the regulation of both innate and adaptive immune response.

ATP released from the dead or injured cells acts as "find me' signal which guides the immune cells to the point of inflammation [123]. Activated T lymphocytes also release ATP in response to antigen stimulation via pannexin channels which activates the P2X7R in an autocrine fashion [23]. This eATP mediated inflammatory response if not controlled will lead to the systemic tissue damage and immunopathology [71]. Therefore, host cells are equipped with ATP degrading surface ectonucleotidases to regulate the hyper-immune response to minimize collateral tissue damage. Treg cells, formerly known as suppressor cells are the subset of CD4 T lymphocytes deputed to protect tissues from the deleterious activities of Th1 cells [124]. Treg cells express abundant CD39 (Nucleoside-triphosphate-diphosphohydrolase-1) and CD73 (5'-ectonucleotidase) on their surface and are found to be strongly up-regulated after antigenic stimulation [125]. The concomitant expression of CD39-CD73 enzyme cascade on Treg cells rapidly strips phosphate from ATP leads to the elevation of local adenosine concentration which suppresses the local Th1 mediated inflammatory response [126,127]. *In vivo* studies demonstrated that CD39 and CD73 deficient Treg cells were inefficient at inhibiting Th1 mediated pro-inflammatory response compared to wild-type Treg cells [48]. The adenosine produced by the Treg acts as a part of an immunosuppressive circuit which blocks the activation of effector cells through A_2Rs activation [107,128]. Th1 cells which lack A_{2A}R or A_{2B}R escape from Treg cell-mediated immunosuppression in various inflammatory and autoimmune models suggests that the Treg mediated immuno-suppressive mechanism principally occurs via the activation of A_2Rs by the extracellular adenosine [129–131].

Several invading pathogens exploit the ATP/adenosine-mediated inflammatory homeostatic pathway to promote disease progression Fig. 2. As macrophages are more prone to eATP mediated apoptosis, extracellular pathogens such as *E. coli* induce the release of ATP, thereby triggers the apoptosis of phagocytic cells and escape from phagocytosis mediated killing [132]. ATP released from activated immune cells trigger a local inflammatory reaction which damages local epithelial tissue that enables the dissemination of pathogen [132,133]. Other pathogens produce toxins such as alpha toxin from *Staphylococcus aureus* (HlyA toxin), beta toxin from *Clostridium perfringens* and pneumolysin from *Streptococcus pneumonia* trigger the release of ATP from the target cells and induce the lysis of the host cells through P2X7R mediated macropore formation in the plasma membrane [134–136]. This toxin-induced lysis of host cell or tissue injury enables the dissemination of pathogen. On the other hand, many intracellular pathogens secrete or express ATP degrading enzymes on their surface to scavenger eATP from the extracellular milieu (Table 3) [137–139]. Some pathogens also induce the up-regulation of ATP degrading CD39 and CD73 enzyme cascade on infected host cells [8,140] and other themselves synthesize and secrete adenosine (catalyzed by bacterial adenosine synthase) into the extracellular milieu which promotes the

local immune suppression and prevents apoptosis of infected cell [138,141,142]. The presence of extracellular ATP not only induces the apoptosis of infected cell but also drives the recruitment of effector cells to the point of infection [23]. Therefore, the clearance of eATP at the site of infection is imperative for the pathogen to establish the infection. Ectonucleotidases promote survival of host cells by degrading eATP thereby delaying the P2X7R mediated apoptosis [143]. This allows intracellular pathogens to divide and perpetuate in the host macrophages. The presence of a large number of infected macrophages increases the probability of parasite transmission to another host when the vectors take the blood meal from the infected host.

Although it is true that by early 1970s the first report on the effect of exogenous nucleotides on macrophage function was published by Cohn and Parks [144]. However, the evidence for the involvement of eATP in the elimination of intracellular pathogens was first demonstrated in 1994 by Molloy *et al.,* in *Mycobacterium tuberculosis* infected macrophages [145]. Numerous studies have since reported the eATP mediated killing of intracellular pathogens. With respect to other ligands that can trigger the lysis of macrophages, including Fas ligation, CD69 activation and complement-mediated cytolysis; the only ligation of eATP to the P2X7R receptors renders the killing of both infected macrophage and intracellular pathogen [72]. The eATP mediated killing of infected cells is basically an innate immune response to circumscribe the dissemination of intracellular pathogens. Earlier it was believed that apoptosis of infected host cell is necessary for the eATP mediated killing of intracellular pathogens [145], however, later Kusner et al., demonstrated that eATP can kill intracellular pathogen through the activation of phospholipase D (PLD) without inducing macrophage death [72]. Activation of PLD is necessary for the killing of intracellular pathogens since inhibition of PLD activation prevents the P2X7R mediated killing of the intracellular pathogen [146]. The absence of PLD activation in $P2X7R^{-/-}$ macrophages confirms the P2X7R mediated PLD activation and intracellular pathogen killing [146]. Similarly, in the absence of exogenous ATP, purified PLD could not clear the intracellular mycobacteria [72]. Further, inhibition of PLD activation rescued the intracellular chlamydiae, but could not prevent macrophage death [146]. These observations suggest that PLD activation via P2X7R required for the elimination of intracellular pathogen.

Stimulation of P2X7R by eATP also leads to the activation of caspase-1, an important mediator of inflammasome activator [147]. Caspase −1 activation is critically required for the proteolytic maturation of pro-IL-1β and pro-IL-18, which plays a pivotal role in NRLP3 mediated inflammation [148]. NRLP3 inflammasome is activated by two mechanisms one by toll-like-receptor (TLR) axis and another by P2X7R activation via eATP [148,149]. Caspase-1 activation is triggered by many pro-inflammatory stimuli such as danger associated molecular pattern (DAMPs) and pathogen-associated molecular patterns (PAMPs), among these eATP is one of the most prominent activators of the caspase-1 [150]. Although PAMPs stimulation such as LPS or bacterial RNA treatment alone elicits IL-1β secretion by TLR activation, however, activation rate is very slow and synthesized IL-β accumulates as an inactive form, however brief exposure of activated cells to ATP enhances caspase-1 activation and IL-1 β maturation [151]. This implies that ATP released from the activated cells through pannexin channel acts as autocrine co-stimulatory signaling via P2X7R stimulation, contribute to the efficient priming of innate immune response along with PAMPs [152–154]. This suggests that host mounts exaggerated inflammatory response to eATP only when a unique molecular signature (PAMPs) from invading pathogen is present, in other words, when the host defence system senses the danger from invading microbes, and then eATP exerts exaggerated inflammatory response. The occurrence of elevated ATP or adenosine concentration in the inflamed tissues, suggests that P2X7R and $A_{2B}R$ are deputed for the regulation of chronic inflammatory response. eATP known to induce pro-inflammatory cytokines such as IL-12, IL-1β, IL-18, TNF-α etc. Production of IL-10 and concomitant suppression of IL-12 is probably one of the central mechanisms whereby adenosine exerts the anti-inflammatory function. IL-10 is the most important cytokine with immuno-suppressive properties virtually produced by all immune cells, circulating monocytes and Treg cells are the principal sources of plasma IL-10. IL-10 exert immune suppression in several ways; inhibits the production of NO, IL-12 and TNF-α from IFN-γ-activated macrophages, inhibits microbicidal activity [155]. Hence, hijacking IL-10 to modulate the host immune system is a prominent feature of many intracellular pathogens [156]. Therefore, the induction of IL-10 by adenosine is one of the immunoevasive mechanisms to stall Th1 development by invading pathogens [8,157,158]. Virtually all immune cells express multiple P1 and P2 receptors, thus extracellular nucleotides affect many facets of immunity and inflammation. In other words, the ratio of extracellular ATP/adenosine represents a possible natural switches that either enhances or suppresses the immune response.

5. Purinergic signalling and pathogenesis of leishmaniasis

Leishmaniases are vector-borne diseases caused by geographically distinct species of the protozoan parasite belonging to the genus *Leishmania* [159]. These obligate intracellular parasites preferentially infect macrophages throughout the skin and viscera, and parasites are usually found in the skin lesions, liver, spleen and bone marrow. The spectrum of clinical manifestation of different forms depends on the phenotypic and metabolic adaptabilities of infecting species of the parasite, infection site, parasite dose and genetic makeup and immunological response of the host [159]. Approximately 53 *Leishmania* species have been described; of these, 31 species are considered to be parasites of mammals, more than 20 *Leishmania* species are recognized as pathogenic for humans and 30 sandfly (genus *Phlebotomus*) species are known as disease-transmitting vectors [160]. Although Leishmaniases are endemic in 98 countries, they are still considered neglected tropical diseases. Leishmaniasis is broadly classified into three clinical forms – visceral leishmaniasis (VL), cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) [159]. Among these, VL is the most severe form of the disease, if not treated; death may occur within few weeks to 2–3 years depending on whether the disease is acute, subacute or chronic [159]. Although VL is mainly found in central Asia, the incidence of VL is relatively low in southern Europe [161], however, the disease has recently spread further northward as shown by recent reports in northern Italy [162], Germany and Spain [163]. Although there are few anti-leishmanial drugs are available, the numbers of VL cases are increasing worldwide, and the enduring problems with current chemotherapy tools are still a critical issue. In the absence of a human vaccine against leishmaniasis, chemotherapy remains the mainstay of disease control. The therapeutic options for VL are limited and far from satisfactory due to low efficacy, toxicity, high cost involved and also the emergence of drug resistance parasites [164]. Thus, there is an exigent need for improved therapeutic interventions against leishmanial infections. Understanding the way by which the immune system responds to leishmanial infection is central to the development of effective control measures.

5.1. Host immune response during Leishmania infection

The host response to leishmanial infection depends largely on the cell-mediated immunity [165]. Progressive leishmaniasis is considered to be due to impaired cellular immunity, with Th1 or macrophage dysfunction or both [166]. The Th1 immune response is critically required for the resolution of *Leishmania* infection; in contrast, the Th2 response is required for maintaining the chronic infection by parasites. Cytokines constitute an important checkpoint for the development of Th1 mediated protective immune response. IL-12, IL-2, IFN-γ and TNF-α are the key Th1 cytokines which are associated with macrophage activation and parasite clearance, on the other hand, IL-10, IL-4 and TGF-β strongly drive disease promoting Th2 type immune response

which is associated with the persistent parasite infection [167]. Earlier work on mice model classically posited IL-4 and IFN-γ as the principal Th1 and Th2 regulating cytokines respectively [168]. However, a seminal work by Noben et al. in IL-4 knockout mice demonstrated IL-4 independent Th2 response during *Leishmania major* infection [169]. The subsequent work in *L. major* infection model demonstrated that *L. major* cause a progressive, non-healing lesion in BALB/c mice deficient for IL-4 [170,171]. Elevated IL-10, IL-4, IL-6, IFN-γ and severely suppressed IL-12 have been observed during leishmaniasis in human clinical samples as well as in the experimental animal models [8,172]. Interference in the IL-10 signalling such as genetic ablation of the *il-10* gene or neutralization of IL-10 or blockade of IL-10R invariably increased the resistance to *Leishmania* infection in susceptible mice, conversely, overexpression of IL-10 renders mice susceptible to *Leishmania* infection [173–175]. On the other hand, IL-12 orchestrates the resistance against *Leishmania* infection by macrophages activation, in turn induction of IFN-γ production, hence, the elimination of intracellular parasites [176]. Genetic disruption of IL-12 or neutralization of IL-12 promotes progressive leishmaniasis in resistance mice [177,178]. It has been observed that the administration of IL-12 at the time of infection prevents Th2 development and protects susceptible mice against *Leishmania* infection [179]. However, administration of IL-12 at the later stage of infection could not reconcile the resistance [179,180]. Therefore, the timing of IL-12 production is also critical in deciding the fate of disease progression. IL-12 initiates the Leishmanicidal function via Th1 cell activation, IFN-γ secretion and granuloma formation. Exogenous IL-12 enhances the therapeutic effect of chemotherapy at a sub-lethal dose [181], whereas exogenous IFN-γ adjuvant therapy could not give an encouraging result in human VL patient [182]. In addition, despite increased plasma IFN-γ level, leishmaniasis patients are unable to resolve the disease [8], even in the Syrian hamster model (which mimic the progressive features of human VL) also elevated IFN-γ is ineffective in mediating exclusive M1 macrophage polarization, restraining parasite replication and disease progression [183]. Therefore, it has been conjectured that the suppression of Th1 effect may be due to the strong inhibitory effects of IL-10 rather than the lack of IFN-γ production [184,185]. To support this notion, previous studies have shown that prior exposure of macrophages to IL-10 prevented them from responding to IFN-γ [167,186], unable to clear the intracellular *Leishmania* parasites after treatment with exogenous IFN-γ [167]. Collectively, these data suggest that the interplay between IL and 10 and IL-12 is one of the factors which govern the Th1/Th2 paradigm and the outcome of the *Leishmania* infection. However, an endogenous mediator which governs the regulation of IL-10 and IL-12 during microbial infection is still debatable. In recent years several novel endogenous anti-inflammatory mediators have been indeed identified. Among them, adenosine emerged as a powerful and evolutionarily selected inflammation modulating factor, which orchestrates the magnitude, duration and resolution of the inflammation [8,187,188].

Elevated plasma adenosine concentration has found to be associated with the immunosuppressive response in both clinical as well as infectious diseases [8,187]. Previously, we have shown that *ex vivo* stimulation of macrophages (monocyte-derived macrophages isolated from Indian VL patients) with adenosine augment the production of IL-10 and suppression of nitric oxide via activation of $A_{2B}R$ [96]. Recently, we have found an association between up-regulated ectonucleotidases (CD39 and CD73) on peripheral blood mononuclear cells (PBMCs) and elevated plasma adenosine concentration, which in turn skews Th1/Th2 balance toward Th2 in Indian VL subjects [8]. Associations between IL and 10 production and accumulation of Treg cells and parasite burden have been observed in the lesions of clinical as well as experimental leishmaniasis [189,190]. Inhibition of A_2Rs activation by different means completely block the Treg mediated immune suppression underlines the adenosine and adenosine receptor-dependent suppression of pro-inflammatory response [107,191]. Significantly reduced IL-10 production and frequency of Treg cells was observed in $A_{2A}R^{-/-}$ BALB/c mice infected with *Leishmania infantum* [107]. Apart from Treg cells, *Leishmania* parasites also induce the expression and activity of ectonucleotidases (CD39 and CD73) on infected macrophages, hence, maintain a constant adenosine cloud at the infection micro-environment and persistence of parasite in the infected tissues [8,158]. Hence, local adenosine concentration at tissue microenvironment acts as immunosuppressive molecule.

In recent past, few studies have reported the occurrence of leishmaniasis in the patients with rheumatoid arthritis (RA) or psoriatic arthritis who are treated with tumor necrosis factor-α (TNF-α) antagonists, corticosteroids or methotrexate [192–195]. Corticosteroids found to exert synergism with $A_{2B}R$ and enhance the immunosuppressive effects of adenosine [196]. It is speculated that the occurrence of leishmaniasis in methotrexate-treated RA patients may be due to the methotrexate-induced endogenous adenosine production [192,195]. To support this notion, experimental evidence from clinical data as well as *in vitro* and *in vivo* studies suggests that the effect of methotrexate is partially mediated by adenosine production [197]. Methotrexate augments the endogenous adenosine production by inhibition of 5-aminoimidazole- 4-carboxamidoribonucleotide (AICAR) transformylase and adenosine deaminase (ADA) both are involved in the catabolism of adenosine [198–200]. These accumulating data imply that extracellular adenosine plays a role in immunosuppression during *Leishmania* infection. It has been found that *Leishmania* infection induces the up-regulation of P2X7 and P2Y2 receptor on host macrophages and both receptors have anti-leishmanial functions once activated by their endogenous agonists ATP and UTP, respectively [201–203]. However, the inability of the macrophage to kill intracellular parasite may be attributed to the degradation of the extracellular ATP and UTP by parasite-induced host ectonucleotidases [8]. In addition to this, ectonucleotidases expressing on the parasites are also involved in the degradation of extracellular ATP and therefore, ectonucleotidases of *Leishmania* parasites are also implicated as one of the virulence factors [28,204].

5.2. Parasite ectonucleotidase activity and immunomodulation

Leishmania species are auxotrophs for *de novo* purine synthesis, they must salvage purine nutrients from the extracellular milieu for their growth and development [205]. Among all nucleosides, adenosine is the most abundant nucleosides found in the extracellular milieu [38] and upon importing into the cytoplasma, adenosine is converted into other nucleobases [206]. Recent studies have shown that triggering of a stress response, depleted ATP synthesis and increased expression of nucleoside transporters occur in *Leishmania* parasite under adenosine-starved condition but not under other nucleoside-starved condition, indicating that adenosine is the most preferred nucleoside for *Leishmania* parasite [207]. Owing to their higher negative charge, nucleotide triphosphates cannot cross plasma membrane, they must convert into nucleosides in order to import into the cells. Therefore, *Leishmania* parasites employ ectonucleotidases on their surface to convert nucleotide triphosphates into corresponding nucleobases [28]. The ectonucleotidases are also acting as an adhesion molecule that facilitates the attachment of parasites to the host cell [208–212]. Since degradation of extracellular ATP is associated with suppression pro-inflammatory response, ectonucleotidases are considered as one of the virulent factors [204]. In line with this hypothesis, virulent *Leishmania* promastigote found to express higher ecto-NTPase activity than less virulent or avirulent strains [8,208,213]. Ecto-ATPase of *Leishmania* has high specificity for ATP compared to other nucleotide triphosphates, this is in consistent with the abundant occurrence of ATP and its derivatives in the extracellular milieu [208,213]. The degradation of eATP by *Leishmania* surface ectonucleotidases is hypothesized as one the mechanism to elude host immune system to avoid apoptosis of infected macrophages and suppression of local inflammatory response [28,201,202]. Apart from surface ectonucleotidases, *Leishmania* parasites also secrete

nucleoside diphosphate kinase (NDPK) and class I nuclease into the extracellular milieu which prevent ATP-mediated cytolysis of macrophages (Table 4) [210,214]. Thus, ectonucleotidase activity not only precludes ATP mediated apoptosis but also promotes the immune suppression via adenosine production. Hence, production of adenosine at the immune-synoptic-junction during *Leishmania* infection is critical for the inhibition of apoptosis, apart from impairing the antigen presentation, production of anti-inflammatory cytokines and suppression of NO and ROS, favoring the persistence of parasites in the infected cells [8,158].

Asides these NTPDases, *Leishmania* parasites also express 3′-ectonucleotidase/nuclease (3′-NT/NU) and 5′-ectonucleotidase on their surface which further degrade AMP to adenosine [215]. 5′-ectonucleotide present on the outer surface of the parasite, involved in the hydrolysis of a variety of mononucleotides [215]. Virulent *Leishmania* strains express higher 5-ectonucleotidase activity compared to less virulent strains and the adenosine generated by this enzyme is implicated in the production of IL-10 [8,216]. On the other hand, 3′-NT/NU of *Leishmania* is a unique enzyme, exclusively present only in Trypanosomatidae family [215]. This enzyme can hydrolyse a variety of 3′-mononucleotides as well as nucleic acids [215]. 3′-NT/NU has 50 fold higher activity than the 5′-ectonucleotidase, expression level is higher in amastigote compared to promastigote stages of the parasite [217]. In addition, while 5′-ectonucleotidase can degrade a variety of 5′-mononucleotides substrates, whereas 3′-NT/NU has higher substrate specificity for adenosine-3-monophosphate (3′-AMP) compared to other nucleoside monophosphate substrates (our unpublished data) implies that this enzymes is mainly meant for the generation of adenosine. Interestingly, 5′-AMP and 5′-GMP substrate of 5′-ectonucleotidase reversibly inhibits the 3′-NT/NU activity [218,219], it suggests that parasite has adapted energy conservation mechanism, when abundant 5′-mononucleotides available it will switch off 3′-ectonucleotidase activity. This enables the parasite to survive in both 5′- and 3′-nucleotide rich environment. Among all forms of leishmaniasis, visceral leishmaniasis is the most severe form of leishmaniasis compared to non-visceral forms. Interestingly, viscerotropic species of *Leishmania* possess significantly higher 3′-NT/NU activity than non-visceral species [220]. Furthermore, 3′-NT/NU is capable of degrading the DNA filament of the neutrophil extracellular trap (NET), hence, escape from NET capturing [221]. In line with this, the higher 3′-NT/NU activity is found to be associated with higher *Leishmania*: macrophage interaction [220]. Since visceral species of *Leishmania* parasites selectively colonizes in liver and spleen, this enzyme seems to have special relevance for the pathophysiology of the leishmaniasis in liver and spleen, because, 3′-AMP the substrate of 3′-NT/NU is abundant in the liver and spleen (3′-AMP approximately 280 nmol/g) compared to other organs [222]. Since host doesn't possess surface 3′-ectonucleotidase it gives an exclusive advantage to *Leishmania* parasites for 3′-AMP substrate. The higher nucleic acid hydrolysis in liver and spleen ensures the surplus substrate for 3′-NT/NU. *Leishmania* parasites also secret nuclease which hydrolyzes a variety of nucleotides as well as nucleic acids; including single-stranded RNA and DNA molecules [210,215]. This secretory nuclease helps in harnessing host-derived nucleic acids at a distance away.

Asides this, *Leishmania* infection up-regulates the surface ectonucleotidase activity on infected macrophages and thereby maintains a constant cloud of adenosine at the vicinity of the infected macrophages [8,158]. This is necessary for the survival and replication of intracellular pathogen [223]. Stimulation of $A_{2A}R$ or $A_{2B}R$ increases the generation of intracellular cAMP leads to the activation of protein kinase A (PKA) which in turn initiates down-regulation of proinflammatory response [101,224]. The activation of $A_{2B}R$ during *L. amazonensis* infection severely dampens the expression of antigen presenting molecules (MHC) and co-stimulatory molecules (CD40, CD80 and CD86) results in the suppression of antigen presentation and maturation of infected macrophages and dendritic cells [158]. Furthermore, activation of $A_{2B}R$ inhibits the production of NO, TNF-α and IL-12 by infected macrophages [211]. Conversely, blockade of $A_{2B}R$ restores the ability of host cells to produce IL-12 during *Leishmania* infection [101]. Recently, Lima et al. demonstrated that $A_{2A}R^{-/-}$ BALB/c mice harbored significantly fewer *Leishmania* parasites in liver and spleen compared to wild-type mice [107]. The reduced parasite load in liver and spleen of $A_{2A}R^{-/-}$ mice is accompanied by reduced IL-10 production and recruitment of neutrophils to the infected organs [107]. The presence of adenosine at the time of *Leishmania* infection augments the host-parasite interaction; increases the tissue parasitism and delays the lesion resolution [204,225]. On the other hand, interference in the adenosine signalling such as the blockade or ablation of adenosine receptor or enzymatic removal of adenosine at the time of infection results in reduced host-pathogen interaction [8,101,107]. These compelling evidence collectively suggest that elevated extracellular adenosine and up-regulated adenosine receptors in leishmaniasis patients promote the disease progression via suppression of Th1 response [8,96].

5.3. Sandfly salivary enzymes

In addition to host and parasite ectonucleotidases, saliva of sandfly also contains apyrase, 5′-ectonucleotidase and pyrophosphatase, and also it is rich in the immuno-suppressive amount of AMP and adenosine [226,227]. These enzymes generate abundant adenosine at the site of sandfly bite results in the suppression of innate immune response [228]. In addition, the optimum activity of salivary apyrase found to be at alkaline pH that corresponds to be the pH of the blood [229]. It is noteworthy to point out that generally 10^6 to 10^7 metacyclic parasites are required for the establishment of artificial infection in the animal models, whereas in the presence of sandfly salivary extract as low as 100–1000 parasites are enough to establish infection in mice [230]. Mice co-infected with *Leishmania* parasite and salivary extract displayed production of a high level of the disease progressing IL-10 and suppression of nitric oxide [231]. This simultaneous surge of IL-10 and suppression of IL-12 is plausibly induced by the production of immunosuppressive adenosine as the sandfly salivary extract contains abundant apyrase and 5′-ectonucleotidase, in addition to AMP and adenosine which collectively enhance the local adenosine concentration at the site of infection [227,232,233]. Salivary nucleotidases act in concert with ectonucleotidases of host and parasite to generate the surplus amount of adenosine at the site of sandfly bite. The early production of adenosine at the site of infection is not only reducing the inflammation but also inhibits the production of NO, platelet aggregation, NET toxicity and prevent the recruitment of effector cells. Therefore, the microenvironment at the site of parasite inoculation profoundly influences the course of the disease outcome [234]. Adenosine may not be the lone source of IL-10 during leishmaniasis, but its presence at the time of host-pathogen interaction exacerbates the disease severity. Adenosine produced at the site of infection may elicit small but enduring immunosuppression on proximate cells which promotes the establishment of chronic infection and progressive leishmaniasis.

6. Challenges in purinergic signalling

Despite significant advances in the purinergic receptor studies in last 20 years, therapeutic targeting of purinergic signalling against infectious diseases is still at its dawn. This is primarily due to the functional redundancy among the purinergic receptor subtypes and promiscuous nature of the existing ligands [235–238]. Due to the wide distribution and remarkable plasticity in the receptor activation, targeting one pathway may directly or indirectly interfere with other ancillary pathways which increase the risk of debilitating side effects. This could be one of the reasons for the failure of synthetic ligands in clinical trials [239,240]. Rolofylline (KW3902) A_1R antagonist, developed against acute heart failure (AHF), however, A_1R also highly expressed

in kidney and critically required in proximal tubule reabsorption [241]. In a large phase III clinical trials, Rolofylline has shown worsening renal function with volume overloaded and kidney dysfunction in AHF patients [242]. In addition, the half-life of the synthetic ligands in the circulatory system is equally critical for the therapeutic efficacy. Endogenous ligands such as ATP, ADP and adenosine rapidly cleared from the system by enzymatic degradation or nucleotide transporters, whereas, synthetic ligands are metabolically stable in the body, hence, could pose sustainable side effects upon long-term exposure.

Purinergic receptors are pharmacologically characterized using either receptor specific antagonist or recombinant receptor expressed in cell lines which precludes the other receptors from the experimental setups [89]. Therefore, more often than not, the phenotypes observed in physiological settings do not match those reported for *in vitro* studies [243]. These poor matches suggest that additional subunits or hybrid receptors might account for these discrepancies. Indeed, the occurrence of novel hybrid receptors have been reported in cell line studies as well as in native tissues [244,245]. The pharmacological properties of each of these hybrid receptors differ considerably from their parental receptor. For instance, the hybrid receptor P2X1/5R found to behave differently from either of the homomer (P2X1 or P2X5) when expressed separately [246]. Since multiple purinergic receptors express on single cells the propensity of heteromeric receptor formation in native tissues is highly possible. Pharmacology of agonists or antagonists of an individual receptor alters when it forms a heterodimer. For instance, in A_1-A_{2A}R heterodimer, activation of A_{2A}R decreases the affinity of the A_1R for its agonist, but not vice versa [121]. Similarly, in A_{2A}R-A_{2B}R heteromeric complex activation of A_{2B}R alters the pharmacology and signalling of A_{2A}R but not vice versa [247]. On the contrary, in A_1-$P2Y_1$ heteromer activation of $P2Y_1$R synergistically activate A_1R [248]. Therefore, in a heterodimeric receptor, one receptor may modulate the pharmacology of its partner without losing its own binding affinity. This phenomenon of “receptor-receptor” interaction significantly influences the potency of the ligand. Therefore, the heteromeric receptor-receptor interaction in the native cells or tissues may alter the therapeutic efficacy of the ligands.

To date, the approach for *in silico* ligand binding data has been based on the crystallographic structures of monomer receptors. Majority of the synthetic ligands have been developed against monomer receptors and evaluated in the cell lines expressing the recombinant receptor [89]. However, such *in vitro* assays exclude the heteromeric receptor, endogenous ligands and ectonucleotidases from the assay system, hence, may exhibit a different therapeutic profile when tested in native tissues [249]. Most of the cells and tissues release nucleotides under a variety of physiological conditions which in turn influence the ligand affinity and affect the activity of the receptor under study [249]. The presence of ectonucleotidases dramatically influences the potency of a ligand under study. A case study of $P2Y_2$R agonist, Denfosol, best illustrates the influence of the ectonucleotidases on ligand potency. The *ex vivo* experimental results demonstrated that the half-life of Denfosol was 25 h [250,251]. However, those experimental settings excluded the endogenous nucleotide metabolizing enzymes. When Denfosol is administered to the volunteers it was metabolized within minutes presumably by endogenous nucleotidases. By the time it was discovered, the program was well into phase 2 clinical trials and ultimately unable to demonstrate any benefits, eventually failed (as a result Inspire Pharmaceutical company lost 400, 000, 000 dollars in a single day). Asides this, the presence of allosteric modulators also influence the ligand potency [61,252]. The allosteric interactions regulate the pharmacological responses by modifying the affinity or signals imparted by orthosteric ligands [253]. Therefore, in the physiological settings, the potency of the orthosteric ligand may vary due to the allosteric modulation. Therefore, one must cautious in extrapolating *in vitro* responses in *in vivo* experimental conditions. The occurrence of different heteromeric partners, the presence of ectonucleotidases, *in vivo* receptor affinity and allosteric modulation should be taken into account for the development of a new therapeutic agent.

7. Opportunities in targeting purinergic signalling

The study of the role of extracellular nucleotides and nucleosides as immunomodulatory molecules has been the focus of several laboratories. Recent developments in purinergic research have greatly increased our knowledge pertaining to purinergic signalling pathways and offer a huge opportunity for the development of therapeutic molecules [254,255]. The crystallographic revolution in the past decade and advent of informatics and molecular modeling tools, combined with *in vitro* and *in vivo* site specific mutational studies disclosed the factors influencing the ligand-GPCR recognition process [256,257]. In addition, modern molecular techniques such as fluorescent resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) approaches have been successfully used to detect the existence of heteromeric receptors in native tissues [121,258,259]. Recent developments in the G-protein coupled receptor (GPCR) structure have provided insight into the allosteric conformational changes that occur during receptor activation. Therefore, these allosteric binding sites can be exploited to enhance the receptor activation at low ligand concentration [32,61].

Apart from synthetic compounds, several promising natural products have been isolated from animal, plant and microbial sources have shown promising agonist or antagonist activities on different purinergic receptor subtypes [260,261]. The scaffolds of these compounds can be exploited to design a novel ligands endowed with greater receptor specificity. Parallely, development of monoclonal antibodies would be a prudent strategy to target the specific receptor subtypes. In this direction, Buell et al. generated P2X7R specific monoclonal antibody which found to inhibit 80% of human P2X7R activity expressed in the cell lines and inhibits the activity of P2X7R agonist BzATP [262]. Interestingly, it could not inhibit the mice or rat orthologs of hP2X7R and other human P2X subtype receptors [262]. Similarly, Shcherbatko et al. generated a panel of monoclonal antibodies against human P2X3R and human P2X2/3 hybrid receptors which produced distinct functional effects depending on the homomeric or heteromeric composition of the receptor [263]. These antibodies would serve as excellent tools to detect heteromeric receptors in native tissues. In another study, epitope-specific monoclonal antibody Adonis was raised against the EL2 epitope of the A_{2A}R, which specifically recognizes a 7 amino acid epitope (LFEDVVP) at the c-terminal loop of the EL2 [264]. *Ex vivo* evaluation revealed that Adonis binds to the EL2 allosteric site and behaves as an A_{2A}R agonist, inhibits T cell proliferation [265]. Recently, Danquah et al. generated P2X7R blocking (13A7) and stimulating (14D5) nanobodies against human P2X7R which bind to hP2X7R with high specificity and showed no cross-reactivity with other human P2XR subtype receptors [266]. Most importantly, these nanobodies have shown long half-life time, up to 4 days *in vivo* condition. Nanobodies are 1/10th the size of conventional antibodies and have a unique propensity to bind functional crevices and epitopes on the protein that are inaccessible to conventional antibodies [267,268]. Owing to their extraordinary specificity, solubility and stability, effective tissue penetration, low toxicity and simple pharmacodynamics, these monoclonal nanobodies emerged as an alternative to the small molecule inhibitors or activators against purinergic receptor [267].

In many pathophysiological conditions, ectonucleotidase derived adenosine has been implicated in the suppression of the immune system via the activation of A_2Rs [8,269]. Therefore, targeting adenosine receptors will be a prudent approach because these receptors are functionally well defined compared to P2XRs and P2YRs types [89]. The local activity of surface ectonucleotidases (CD73 and CD39) found to be up-regulated on the immune cells at infection and inflammatory settings, and is complemented by parallel increase in the expression of A_2Rs [8,47,48,96], making it a preferential target for the site-specific or event specific delivery of drugs or pro-drugs. Recently, Ulrich Flögel

et al. ingeniously designed a synthetic phosphorylated $A_{2A}R$ agonist, 2-(cyclohexylethylthio) adenosine 5′-monophosphate (chet-AMP), a prodrug which requires the presence of 5′-ectonucleotidase (CD73) to become a bio-active drug. Upon systemic administration in collagen-induced experimental rheumatoid arthritis in mice model, the prodrug is preferentially cleaved by CD73 at the site of inflammatory joints and achieved a potent anti-inflammatory activity with negligible vasodilatory activity [270]. Such ingenious methods of site-specific or event specific drug delivery can fend off the unfavorable side effects due to the systematic accumulation of adenosine and its analogs. In the same line, by virtue of its T cell proliferating ability and adenosine deaminase activity, ADA1 can be used as the immune-stimulating agent. Therefore, recombinant ADA1 can be used as a T cell stimulant and to clear the adenosine cloud at the immune-synoptic-junction to prevent adenosine-mediated immunosuppression [271]. Therefore, human ADA1 can be engineered to enhance its affinity for CD26 as well as an enzymatic activity to clear adenosine from the cell periphery at immuno-synaptic-junction. Such ingenious approaches can considerably reduce the sustained side effects associated with purinergic signaling.

8. Future prospects

Despite having neglected for decades, purinergic receptors are regaining interest from immunologist as new therapeutic interventions. The recent developments in purinergic receptor structure and functions ably combined with modern tools and technologies have catapulted the designing of purinergic receptor-based therapeutic drugs [257]. Global approval of Clopidogrel ($P2Y_{12}R$ antagonist anti-thrombotic drug), Diquafosol (a long-acting $P2Y_2R$ agonist) and Istradefylline ($A_{2A}R$ antagonist for the treatment of Parkinson disease) has provided a considerable impetus for the development of new classes of therapeutic agents [272,273].

Ectonucleotidases from various pathogens have already been shown to involve in the invasion. Yilmaz *et al.*, demonstrated that targeting of ectonucleotidase of invading pathogen blunts the infectivity of the pathogens and ultimately elimination from the host cell [143]. It is equally evident that targeting P2X7R or A_2 adenosine receptors ($A_{2A}R$ or $A_{2B}R$) of host augment the clearance of intracellular *Leishmania* parasite [80,101,107]. Therefore, ectonucleotidase of *Leishmania* parasite could be an “achilles heel” for leishmaniasis. In this context, 3’-NU/NT could be targeted, as the homologs of this enzyme is completely absent in mammalian host, hence, pose untoward effects on the host [215]. Targeting $A_{2B}R$ will be another prudent approach because it is up-regulated on infected macrophages; therefore, $A_{2B}R$ would be a preferred target for site-specific $A_{2B}R$ antagonist delivery [96,158]. Like mammalian hosts, parasites also express P2X receptors which have different pharmacological properties compared to mammalian P2X receptors [274–276]. The P2X receptor of parasites is reported to generate second messengers that increase the cytosolic calcium upon exposure to extracellular ATP which can be reversed by purinergic antagonists [277]. Since P2XRs of parasites have different sensitivity to endogenous ligands this may have potential therapeutic implications [274]. Indeed, Figliuolo et al., have shown that direct treatment of *Leishmania amazonensis* with periodate-oxidized ATP (oATP), an antagonist of P2X7R resulted in impaired parasite growth with altered morphological and functional changes [278]. This implies that purinergic pathways of parasites can also be exploited for the designating of new anti-leishmanial drugs. Despite the wealth of existing data on the role of adenosine and ectonucleotidases in *Leishmania* pathogenesis, clinical manipulation of purinergic signalling against leishmaniasis is in its infancy. The purinergic signalling during infection is a double-edged sword, a great deal of comprehensive and unambiguous characterization of nucleotide receptors is required still to establish the complex signalling of each receptor in the regulation of the host-pathogen interactions. Therefore, understanding the consequences of pathogen-mediated manipulation of host purinergic signalling may potentially translate the purinergic signalling into a feasible form of treatment.

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Conflict of interest discloser

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