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Purinergic signaling during intestinal inflammation

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Introduction

Inflammatory bowel disease (IBD) is an important illness of unclear pathogenesis associated with major defects in mucosal immunoregulation and develops in genetically susceptible individuals. These abnormalities often occur in association with microbial dysbiosis and result in unfettered inflammation of the intestine and extraintestinal tissues. Such events result in long-term morbidity and possibly even death, in otherwise healthy adults and children.

Dampening inflammation and re-establishing immune tolerance in IBD remain the major therapeutic goal. However, existing IBD therapies albeit providing recent advances, still largely rely on broad-based immunosuppression. For example, only around half of the patients treated with anti-TNF agents show substantive clinical responses. These improvements are often self-limited, while unfortunately increasing the risk of opportunistic infections.

The goal of our laboratory has been to investigate the control of mucosal immune responses, which are based on fundamental signaling pathways. Our own long-term interests in the regulation of purinergic signaling are now being leveraged to develop innovative and hopefully non-toxic therapies for IBD. This review and the accompanying articles in this special issue address new therapeutic concepts in IBD, as based on recent, linked work in hypoxia and purinergic signaling, mucosal barrier functions and microRNA biology.

In several recent, comprehensive reviews (1-4), we have already addressed the biological functions of ecto-enzymes, such as CD39, CD73 and CD38, in the regulation of purinergic signaling and control of extracellular adenosine levels. Others, and we, have noted the

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importance of these mechanisms in immunomodulation, as in cancer and inflammation. The ectonucleotidases of the CD39 family, in particular, have major impacts on the dynamic equilibrium of proinflammatory extracellular ATP, ADP nucleotides *vs*. the immunosuppressive potential of adenosine nucleosides. CD39 plays a dominant role in purinergic regulation of vascular inflammation, thrombosis and the immune response in such settings. As such, the relevance and importance of these purinergic signaling pathways in selected neoplastic states (lymphoma and chronic leukemia) and inflammatory diseases (sepsis and autoimmunity) have been already alluded to in recent work.

In this update, we first provide a brief synopsis of the major components of purinergic signaling; chiefly for those not familiar to this field. We will focus on very recent work detailing the immunomodulation of CD39 on T cells and other immune cells by both genetic and environmental factors in the setting of IBD and experimental colitis, inclusive of the new roles for natural metabolites such as bilirubin. We will also briefly cover the role of CD39 expression on exosomes and microparticles, in control of inflammation in the gut and touch on the relevance of the microbiome. Lastly, we cover the emerging importance of other NTPDases of the CD39 family and speculate on their role in controlling gut inflammation.

Overview of Purinergic Signaling

Extracellular nucleotides (e.g. ATP, UTP, ADP, NAD), and the derivative nucleosides (e.g. adenosine from ATP), are released in a regulated manner by most cells to provide the initiators and primary components for purinergic responses (5). In such settings, this process involves pannexins, which are conserved transmembrane channels that allow the passage of ions and small molecules. The pannexin-1 channels, as an example, mediate the release of ATP from activated T cells and dendritic cells; or even operate following apoptosis.

Under conditions of inflammatory stress, much higher levels of ATP and other nucleotides or nucleosides are released to the extracellular space by pannexins, gap junction hemichannels e.g. connexin 43, following exocytosis as from dense platelet granules, and with active cell death. These extracellular nucleotide/nucleoside mediators bind specific purinergic receptors, which comprise an essential requirement for this signaling network.

Almost all immune and vascular cells express multiple type-2 purinergic/pyrimidinergic (P2) receptors for nucleotides and adenosine or type-1 purinergic (P1) receptors (6). There are at least seven ionotropic (P2X1-7), eight metabotropic ($P2Y_{1,2,4,6,11\text{-}14}$) and four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, A_3), which have been identified (more if one tallies in heteromers) (7). P2X and P2Y11 receptors are chiefly activated by extracellular ATP; P2Y2 by ATP and UTP; P2Y1, P2Y12 and P2Y13 by ADP; P2Y4 by UTP; P2Y6 by UDP and P2Y14 by UDP-glucose. (Nomenclature: http://www.guidetopharmacology.org/targets.jsp).

Extracellular ATP and the related nucleotide derivatives play important roles as signaling molecules. These pro inflammatory mediators participate in both autocrine and paracrine circuits to regulate cellular metabolism, migration, proliferation and apoptosis through signaling pathways triggered by P2Y and P2X receptors. Extracellular nucleotides also serve

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as substrates for ectonucleotidases, which generate the immunosupressive nucleoside, adenosine, after phosphohydrolysis via the ecto-enzymes on the cell membrane with catalytic domains located in the extracellular compartment.

CD39 is the prototype of the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family (EC 3.6.1.5). These proteins comprise a group of ecto-enzymes that hydrolyze extracellular nucleoside tri- and diphosphates. One important ecto-nucleotidase chain or cascade, is initiated by these NTPDases, and is then terminated by ecto-5′-nucleotidase (CD73; EC 3.1.3.5) (8, 9).

As another example, AMP can be phosphorylated by ecto-adenylate kinase, or dephosphorylated by ecto-alkaline phosphatase (1, 9). These and other ecto-enzymes hydrolyze extracellular nucleotides to generate nucleotides and nucleosides, which in turn differentially activate other P2, and then ultimately adenosine receptors. Whereas extracellular ATP generally provides pro-inflammatory signals, the extracellular adenosine produced from ATP/ADP/AMP degradation has potent immunosuppressive effects mediated by adenosinergic responses and cAMP-mediated effects. Hence, events triggered by adenosine generation may often have opposing effects to those seen with the initial P2-mediated effects (10).

Other cell surface-located nucleotide hydrolyzing and interconverting ectoenzymes have been described. These include the ecto-nucleotide pyrophosphatase phosphodiesterases (E-NPPs; EC 3.1.4.1, EC 3.6.1.9 and the autotaxin group), CD38, NAD-glycohydrolases, alkaline and acid phosphatases, diadenosine polyphosphate hydrolases, adenylate kinases, nucleoside diphosphate kinase, and potentially ecto-F1-Fo ATP synthases (9). These other ectonucleotidases are not covered in detail here, given space constraints.

Recent work has also shown that E-NPP1 appears to catalyze transformation of NAD and ADP-ribose to generate AMP (11). Moreover, it is also apparent that ecto-5′-nucleotidase/CD73 together with adenosine deaminase-1 and 2 (ADA1 and 2 (latter only in man, as no orthologous mouse gene exists); EC 3.5.4.4 and 3.5.4.2) generate and then convert adenosine to inosine. These pathways closely regulate local, pericellular and extracellular concentrations of adenosine; and are also required for intracellular salvage with synthesis of nucleotides derived from intermediates produced by these and other degradative pathways (9).

In the short-term, P2R ATP-mediated effects and the linked signaling pathways receptors trigger sudden patho-physiological processes, impacting acute inflammatory processes. Ongoing activation of P2R-signaling pathways impact later immune responses resulting in chronic inflammatory disease and exacerbating fibrosis (3, 12). Immunosuppression in the short term is mediated by generation of adenosine; whereas the fibrosis seen in chronic inflammation may be linked to unfettered adenosine generation. The challenge is to dissect out beneficial effects of adenosine, preclude resistance to these vs. preventing the deleterious signaling pathways of adenosine that cause chronic disease. Aberrant control of these purinergic activities could impact inflammation as well as fibrogenic reactions, as in chronic disease processes such as IBD.

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Cellular Immunomodulation and CD39

There is evidence that innate and adaptive immunity can be modulated by extracellular adenosine, released in response to tissue disturbing signals and extracellular nucleotides such as ATP or nicotinamide adenine nucleotide. Upon binding to multiple type 2 purinergic/pyrimidinergic (P2Y and P2X) receptors, ATP can have effects on cellular metabolism, migration, proliferation and apoptosis. Transcriptional upregulation of CD39 and CD73 ectonucleotidases with increased immune cell infiltration at sites of injury results in conversion of a dominant P2-environment to one associated with decreased levels of nucleotides and a shift over to more predominant adenosinergic responses.

CD39 is highly expressed on vascular endothelial cells and T regulatory cells, where this ecto-enzyme contributes to suppressive functionality through the generation of adenosine (13) (see Fig. 1). CD39 is also expressed by subsets of memory cells with effector function (14) and by M2 anti-inflammatory monocytes (15).

Further, CD39 induction on prototypic, pathogenic Th17 cells imparts regulatory properties to these cells. These transitioned Th17 cells express CD39 and select functional features of T-regs, including expression of FOXP3 at high levels and suppression of responder cell proliferation and pro-inflammatory cytokine production (16).

Despite acquiring regulatory features, these ‘suppressor-like’ Th17 (supTh17) cells also retain certain effector Th17 cell properties, including IL-17 production and low levels of A2A adenosine receptor. Because of heightened expression of adenosine deaminase, these suppressive Th17 cells effectively hydrolyze the nucleoside adenosine into the somewhat more pro-inflammatory inosine derivative and hence appear to exhibit a dualistic phenotype. Of note is that inosine can activate A3 receptors to produce mast cell degranulation, which further regulates the chemotaxis of neutrophils and macrophages (17, 18).

Previous studies from Esplugues and colleagues have shown that pathogenic Th17 cells undergo “regulation” in the small intestine. Indeed, while still expressing IL-17A and IL-17F, these cells also become capable of producing IL-10 and of “suppressing” responders.

Our own evidence that these cells maintain classical Th17 features while acquiring typical T-reg properties, inclusive of CD39 expression, indicates that this lymphocyte subset may exert dual function depending on the environment within which it operates. However, it should also be noted that extracellular nucleotides may serve as negative modulators of immunity, or as immunodepressants. Indeed, chronic, repetitive exposure to lower extracellular nucleotide levels tends to suppress immunity and inflammation (19).

Lastly, our studies have indicated that altered CD39 expression and changes in the nucleotide/nucleoside balance impact insulin-sensitivity, block mTOR activation (ATP-dependent) while boosting AMPK functions (adenosine-dependent process) (20). Although CD39 appears to be associated with enhanced T cell survival, much as rapamycin and metformin are known to do so, additional effects of CD39 include protection from P2X7-mediated apoptosis and the provision of nucleosides that activate A2A receptors, obviating

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activation induced cell death (AICD), promoting intracellular anabolic as well as purine salvage pathways.

CD39 and regulation by the aryl hydrocarbon receptor (AHR) and HIF-1 alpha

The aryl hydrocarbon receptor (AHR) is ubiquitously expressed cells and specific patterns of activation upregulates E-NTPDase-type ectonucleotidases on immunocytes, myeloid cells, endothelium and parenchymal cells *in vivo* and *in vitro* (21).

The ligation of AHR by dioxins in the presence of TGF-beta induces $Foxp3^+$ inducible T-regs that can suppress responder T-cell functions via CD39 (22).

Activation of Ahr can promote generation of CD39+ regulatory-type T helper type 17 (Th17) cells as well as type 1 regulatory T cells or Tr1 cells, which express high levels of IL-10. Upregulation of CD39 is dependent upon ligation of the AhR on immune cells. AhR is additionally controlled by hypoxia and HIF-1 alpha activity, as in the case of Tr1 cells (23). Furthermore, hypoxic conditions per se might activate the purinergic signaling by up-regulating expression of CD39, as shown in the cardiac ischemia model in which transcription of CD39 was controlled by Sp-1 (24), and through HIF-1 alpha induction of CD73, which ultimately converts AMP into adenosine (25).

Recent elegant work has shown that adenosinergic A2BR-mediated responses, which are anti inflammatory and cytoprotective involve further interactions of HIF-1 alpha and the circadian rhythm protein PER2 (26, 27).

Other groups have also shown that the alternative adenosinergic A2AR pathway, together with TNF, have the capacity to regulate immune cell intrinsic “clocks” implicating involvement of circadian rhythms in clinicopathologic changes in prototypic rheumatological disease, as with morning stiffness (28).

Furthermore, there are important seasonal and latitudinal patterns linking IBD exacerbations to light exposure and circadian rhythms. There are substantive differences in the expression of circadian-type genes between normal and diseased intestinal mucosa in IBD. Such deregulated genes e.g. PER1 and PER3 could have pathophysiological relevance and may suggest novel therapeutic approaches distinct from the facile use of melatonin in such disease settings (29, 30).

In the past few decades, bilirubin, a byproduct of heme catalysis, and a pigment also clearly altered by light exposure, has been shown to have a major salutary role as a potent antioxidant. Most recently, the molecule has been found to possess immunomodulatory properties that rival the redox capacity. These possibly explain its ability to suppress inflammation as in IBD, where development of jaundice has been shown to suppress colonic inflammation. We have recently demonstrated unconjugated bilirubin to serve as a potent immunomodulator and have shown that the molecular basis for its immunosuppressive effects is dependent upon the upregulation of CD39 by interactions with AHR (31) (Longhi et al., 2017, in press and Fig 2).

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Hence, limitations in the levels of CD39 and/or dysfunction of AHR abrogate the protective effects of unconjugated bilirubin in experimental colitis and in IBD patients. Therefore, in DSS-induced colitis, the administration of unconjugated bilirubin systemically resulted in amelioration of disease activity particularly during recovery, improved histology scores, and increased IL-10 production by colonic intra-epithelial CD4 cells. These salutary effects were abrogated in *Entpd1-/-* and AhR^{d} mice, in which AhR is dysfunctional (32). Notably, unconjugated bilirubin fails to boost CD39, FOXP3 and immunosuppressive function in IBD derived Th17 cells, which additionally display defective AhR bioactivity (Longhi et al., 2017, in press). The concept that beneficial effects of AhR ligation are mediated via CD39 induction has been also supported by recent work by Goettel et al in the context of experimental colitis (33). Administration of ITE, another AhR endogenous ligand, prevents T-cell mediated tissue damage in humanized mice. This effect is associated with an increased proportion of $CD39^{+}$ CD4 lymphocytes sequestrated in the colonic wall compartment.

Overall, these findings suggest that boosting AhR signaling upon exposure to natural/endogenous ligands or otherwise enhancing CD39 ectoenzymatic properties might represent attractive strategies to correct effector Th17 dysfunction in IBD.

Purinergic/Adenosinergic Responses in IBD and Experimental Colitis

Given the immunosuppressive properties of adenosine, modulation of purinergic signaling has been evaluated in the context of IBD and experimental models of colitis, to curb inflammation. The suggestion that adenosine might be a key immune mediator controlling inflammation in IBD, was indicated first by mechanistic studies of sulfasalazine, and methotrexate. Both drugs have been shown to be operational, at least in part, through adenosine-dependent mechanisms.

Furthermore, administration of ATL313, and other direct agonists of the A2A adenosine receptor can attenuate colitis in mice with adoptive transfer of $CD45RB^{high}$ cells, and also suppress the production of pro-inflammatory cytokines (IL-2, IFNγ, and TNFα) but not the anti-inflammatory (IL-10 and TGFβ) cytokines (34).

Also, albeit controversial, direct activation of the A2B adenosine receptor can also boost IL-10 release by intestinal epithelial cells, which is linked to amelioration of DSS colitis (35).

Alterations in the generation of adenosine, such as those associated with genetic deletions of CD39 or CD73, result in more severe course of experimental colitis in mutant mice. CD39 deletion in mice results in exacerbation of DSS-induced and other experimental colitis, whereas transgenic over expression appears to ameliorate disease (unpublished observations Maria Serena Longhi and Simon C. Robson; 2017) (36).

Expression of CD39 on endothelial or immune cells allows for homeostatic integration of immunity resulting in control of hemostatic and immunobiological reactions, which appear to be disrupted in IBD. Single nucleotide polymorphisms adjacent to the CD39 promoter region have been associated with low levels of CD39 mRNA that confer susceptibility to

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Crohn's disease (37). The associated decreases in CD39 expression levels and consequently lower adenosine generation are likely to be linked to the impairment of $CD4^{+}CD25^{high}$ regulatory T cells in this disease process.

Since this publication in 2009, it has been increasingly recognized that highly heritable traits that dictate adaptive immune responsiveness include the different levels of expression of CD39 on Treg, as noted in large population analyses and twin studies (38, 39). In contrast, CD73 expression by Treg, in humans seems to be at least in part a consequence of the environmental exposure to e.g. pathogens, diet or microbiome elements, shared in a household during maturation (40). This recent work is suggestive of adaptive immune traits being more impacted by genetics, while in contrast innate immune traits are dictated more by environmental factors. Irrespectively, intrinsic or acquired defects resulting in lower levels of CD39 might lead to T cell autoreactivity because of the lack of immune-modulatory adenosine.

CD39 and Exosomes in IBD

Microparticles (exosomes or extracellular vesicles; MPs) are released from cells into the blood or at sites of inflammation in the intestinal tract. These MPs can be isolated from blood, tissue fluids or fecal samples. Depending on the cellular origin, intestinal MP express cell surface markers and contain protein / RNA with pro- or anti-inflammatory properties. In addition, these MP constitute a mode of communication through which intestinal cells may influence the luminal microbiome. We have recently reported that extracellular vesicles, derived from the colonic luminal fluid of IBD patients, display pro-inflammatory properties as these MP contain high mRNA and protein levels of IL-6, IL-8, IL-10 and TNF-α, and promote macrophage migration (41). We have also shown that CD39 associates with circulating plasma-derived MP and may directly or indirectly confer functional properties on cells.

Indeed, surrounding cells can absorb MPs shed from sites of inflammation. We have demonstrated the presence of E-NTPDase activity in circulating MP isolated in human plasma (42). Most importantly, the mRNA within MPs can be taken up by these cells and further translated. We have recently shown that properties of MPs obtained from patients with IBD provide a mechanism for some of the regional variations in inflammation, as noted within the diseased intestinal tract. We have also shown modulatory roles for CD39 within MPs in the exchange of regulatory signals between leucocytes and vascular cells (43).

Particular interest exists in programming cell lines to produce MPs with phenotypic characteristics, such as IL-10 induced anti-inflammatory CD39 expressing MPs from dendritic cells. Our own work proposes that intrinsic properties of MPs suggest a role as novel biomarkers of inflammatory pathways, or even as therapeutic vehicles for local delivery of anti-inflammatory compounds and purinergic modulators in IBD, as we have previously determined in liposomal reconstitution of CD39 (44).

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Microbiome elements - fecal transplants to correct dysbiosis and aberrant purinergic signaling

The commensal flora is recognized to play an important role in the control of the immune response in the context of IBD and experimental colitis (45). Different molecules mediate effects of the microbiome on the immune response, including long-chain fatty acids and tryptophan derivatives that also trigger AHR. Extracellular ATP released by commensal bacteria has been shown to activate purinergic inflammatory signaling to promote the differentiation of intestinal Th17 cells (45, 46).

The NLRP3 inflammasome catalyzes the production of active IL-1 and IL-18 in response to diverse endogenous or exogenous danger signals. One such signal is ATP, which activates the NLRP3 inflammasome in DCs through a mechanism mediated by P2X7R (47).

Curiously, the derivative adenosine alone can also activate the A2AR/CREB/HIF-1 alpha pathway, which is also required for sustained production of IL-1 after the initial inflammasome activation (48).

The importance of the NLRP3 inflammasome in the T-cell response can be highlighted by the decrease in Th1 and Th17 responses observed in NLPR3-deficient mice. CD39 also appears to impact the NLRP3-associated control of T-cell immunity, as recently shown by collaborative studies of tolerogenic DCs induced with IL-27 (23).

Conversely, several pathogen bacteria express ectonucleotidases that may modulate the immune response through the effects on purinergic signaling. Several bacteria also release factors that induce CD39 expression on immune cells (49). Taken together, these findings suggest that extracellular ATP and derivatives produced by microorganisms and by host cells in response to microbial molecules, such as TLR agonists, might play an important role in dictating the relationship between the host and the commensal flora.

This topic addressing the role of ectonucleotidases on host-pathogen interactions has been previously reviewed in Samson et al (50).

It is generally accepted that the microbiome in IBD, in particular in Crohn's disease, is characterized by reduced diversity, particularly of Firmicutes and Bacteroidetes. We recently conducted an open label study transferring the intestinal microbiota from healthy individuals into patients with IBD in order to see if this could correct dysbiosis and reverse mucosal inflammation. Those patients who had clinical responses demonstrated significant shifts in fecal microbial composition toward the respective donor's profile and we also noted an increase in Treg in this subset (51). Further work is ongoing to dissect out the nucleotide and purine metabolome in these patients post fecal transplant and microbiome transfer.

Recent work has shown potential relevance of the mycobiome in colitis (52). *Saccharomyces cerevisiae* has been recently shown to both exacerbate experimental colitis and increase gut barrier permeability in mice. Yeast colonization was found to enhance host purine metabolism in germ free animals, leading to an increase in uric acid production. Importantly, treatment with uric acid alone worsened disease and increased gut permeability. This

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interesting area of research is somewhat controversial given that *Saccharomyces cerevisiae* can be also considered as a probiotic as it may limit adherent-invasive Escherichia coli (AIEC) in CEACAM6-expressing mice (53).

Other NTPDases expressed in the gastrointestinal tract and putative roles in IBD

The ecto-ATPase activity in the gut predominantly resides in blood vessels, immune cells, visceral smooth muscle, and the enteric nervous system (54). While CD39 is the major E-NTPDase expressed by the endothelium and immune cells, we have noted that NTPDase2 and NTPDase3 are responsible, in large part, for the ATPase activity in the muscle layers and the nervous system. In addition, Kusu and coworkers have reported the expression of NTPDase7 by the epithelial cells of the murine small intestine (55).

NTPDase2 and -3 are two cell membrane located ecto-enzymes in the E-NTPDases family that share significant structural homology and functional similarity to CD39 (56, 57). The enzymatic activity of NTPDase3 is similar to that of CD39, whereas NTPDase2 has significantly weaker ADPase activity (58). NTPDase7 is also known as LALP1, and is conventionally thought to be an endo-apyrase. Whether it is also expressed on the plasma membrane in humans is yet to be fully confirmed (59).

Both NTPDase2 and -3 are known be expressed in nerve tissues (60, 61). In the gut, the expression of NTPDase2 has been further noted on glial cells, while NTPDase3 localizes to both glia and neurons (54, 62, 63). In both humans and mice, NTPDase3 antibodies positively stain nerve fibers penetrating the smooth muscle layers, whereas the expression of NTPDase2 in these areas is less prominent (Feldbrügge et al., 2017, in press and see Fig. 3).

We have further shown that the genetic deletion of *Entpd2* results in exacerbated DSS-induced experimental colitis in these mutant mice that do not express NTPDase2. This outcome is associated with an increase the proportion of proinflammatory macrophages in the lamina propria. Similarly, mice globally null for *Entpd3*, which lack all NTPDase3 expression, have more pronounced anemia compared to wild type in this same DSS-induced colitis model. We have also compared the ADPase activity in the plasma of patients with Crohn's disease and controls, and found that Crohn's patients have lower circulating ADPase activity. This ADPase activity is in part contributed by non-CD39 NTPDases, as suggested by sensitivity to non-CD39 NTPDase inhibitors.

The emerging roles of NTPDase2 and -3 in IBD further support the innovative concept of neuro-immune interaction (64), (65). This interaction may function at multiple levels. Hence, eATP transmits signals both amongst neurons in myenteric and submucosal ganglia via P2X2 and P2Y1 receptors and between nerve and smooth muscle cells via P2Y1 receptors, exerting an inhibitory effect on the muscularis (66-68). eATP can also activate ionotropic P2X7 receptors in macrophages, dendritic cells and neutrophils, which in turn induces NLRP3 inflammasome assembly and the release of interleukin 1β and 18 (69). Furthermore, extracellular ATP has been shown to mediate the communication between neurons, glia, and contribute to the maintenance of intestinal homeostasis and mucosal

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barrier (70) (71). Glial cells can perpetuate the release of pro-inflammatory ATP in the setting of intestinal inflammation via the activation of P2Y1 receptors, which in turn mediates neuronal cell death via P2X7 receptors (72). A recent study by Gabanyi and coworkers also suggested that enteric neurons in the muscularis externa can mediate the polarization of tissue resident macrophages toward a tissue-protective phenotype (65). Purinergic signaling may be a crucial mechanism modulating this interaction.

Interesting work from Kusu et al. with respect to NTPDase7, suggested that there is yet another mechanism that gut purinergic signaling can modulate host immunity (55). The team at Osaka University observed that NTPDase7 expressed on the intestinal epithelial cells modulates the ATP content in the intestinal lumen per se. Mice null for Entpd7 and deficient in NTPDase7 cannot scavenge luminal ATP produced by commensal microbiota. As a consequence, this enhances the development of proinflammatory Th17 cells, leading to a more severe phenotype in models of experimental autoimmune encephalomyelitis. Whether such immune dysregulation is relevant to human IBD remains to be determined.

Conclusions

This manuscript has summarized the role of aberrant purinergic signaling in IBD and gastrointestinal autoimmunity and has suggested how pharmacological modulation of purinergic responses, adenosine generation, AhR and HIF-1 alpha signaling (amongst others) could be exploited to treat these important conditions. The purinergic signaling pathways could be targeted for IBD treatment by the use of soluble ectonucleotidases, adenosine receptor agonists, or HIF activators, inter alia; as previously addressed in two important reviews (73, 74):

In this review, we have highlighted how targeting CD39 (and related ectonucleotidases) to modulate the purinergic-adenosinergic axis could have major impacts on extent of the inflammatory infiltrate in IBD (via adenosine receptor agonists and/or boosting CD39 or related ectonucleotidases). We propose that augmentation of CD39 and related ectonucleotidase bioactivity, possibly in MPs, might also control aberrant autoimmune reactions (via pharmacological use of adenosine receptor agonists and/or regulated CD39 expression).

These purinergic mechanisms involved in both the generation of adenosine and scavenging of extracellular nucleotides have major impacts on the downstream signaling pathways critical to both thromboregulation and most importantly to the progression of inflammation and are hence of considerable and increasing therapeutic interest.

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Nomenclature/abbreviations: List and define any unusual symbols used in your article

ACR	apyrase conserved regions
AhR	aryl hydrocarbon receptor
DC	dendritic cell
EC	endothelial cell
E-NTPDases	ecto-nucleoside triphosphate diphosphohydrolases
IBD	inflammatory bowel disease
IL	interleukin
NPP	nucleotide pyrophosphatase/phosphodiesterase
Treg	T regulatory cells
Tr1	type 1 regulatory T cells

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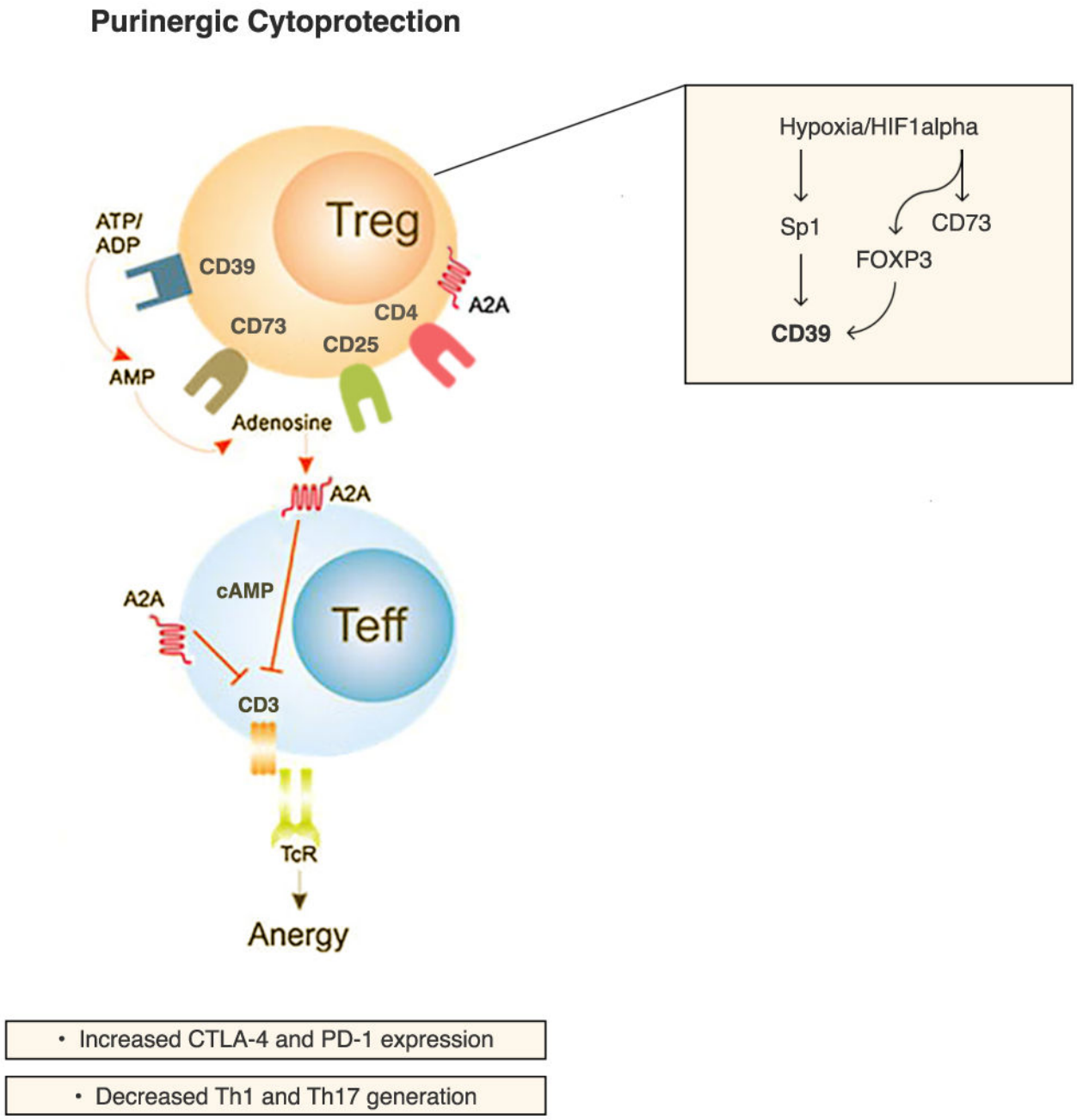

Fig. 1. Purinergic cytoprotection

This illustrates the role of the purinergic ecto-enzyme network in gastrointestinal inflammation. T regulatory cells (Treg) express the entire ecto-enzymatic machinery necessary to convert ATP/ADP into adenosine. Increased extracellular adenosine levels contribute to creating a favorable, homeostatic microenvironment by switching off T cell responses, producing anergy, inducing cytoprotection in an autocrine fashion, and promoting resolution of inflammation in the gut. Note also that hypoxia/HIF-1 alpha may modulate FOXP3; as well as CD39 and CD73 expression via Sp1. See text for details.

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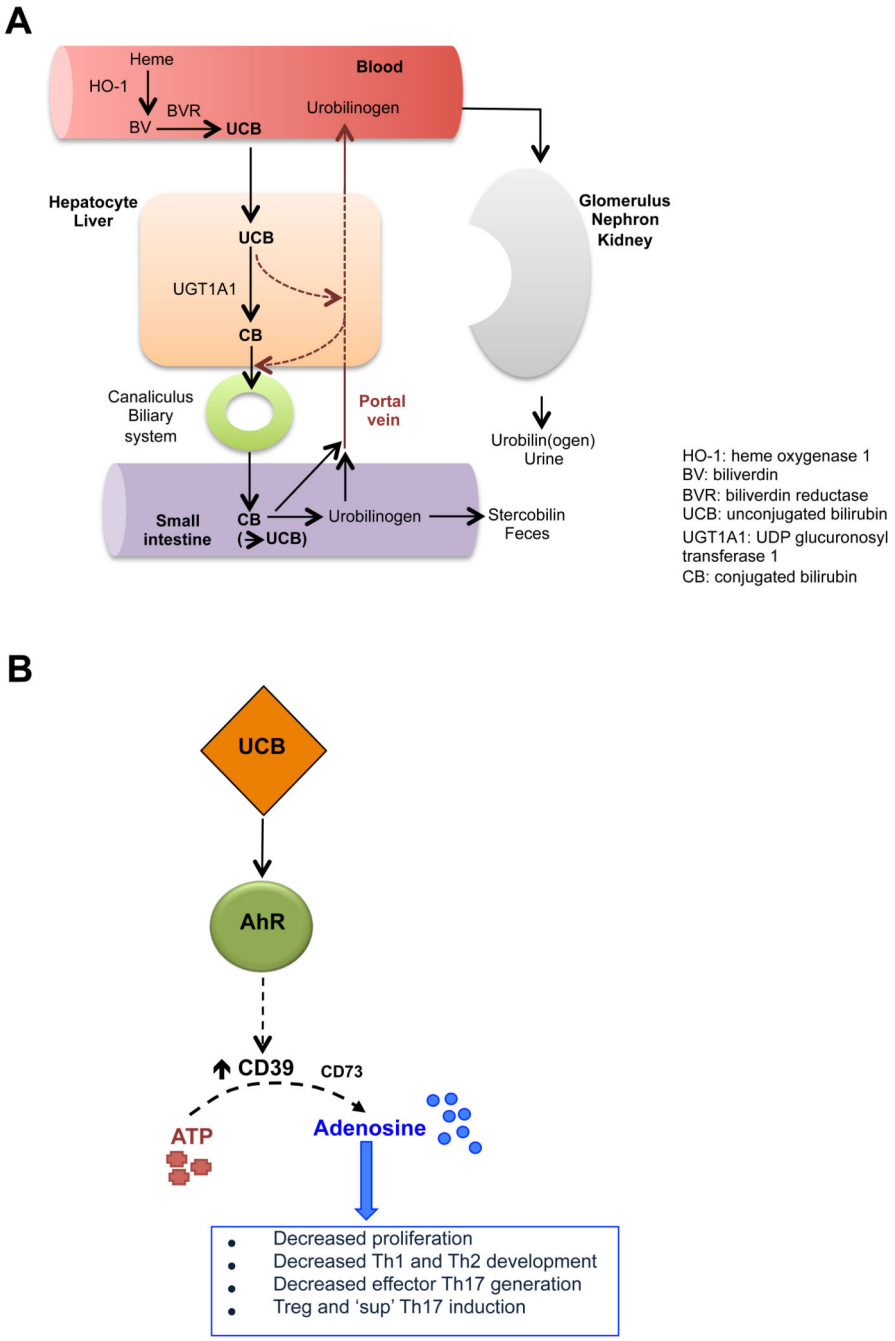

Fig. 2. Bilirubin metabolism and mechanism of action

(A) Unconjugated bilirubin (UCB) is an end product of heme catalysis and has known immunosuppressant properties Heme-oxygenase-1 (HO-1) catalyzes heme degradation to biliverdin (BV), which is then converted to UCB by biliverdin reductase (BVR). As it is insoluble in water, UCB binds to albumin in the circulation. In the hepatocytes, UCB is conjugated with glucuronic acid by UDP glucuronosyltransferase 1 (UGT1A1) and is then excreted into the bile. Then, after being metabolized to urobilinogen and de-conjugated by

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the bacterial flora, it is excreted in the urine and feces. Proportions of bilirubin are, however, re-absorbed and undergo enterohepatic circulation.

(B) UCB serves as an endogenous ligand for the aryl hydrocarbon receptor (AhR), a mediator of toxin responses and adaptive immunity. AhR engagement by UCB results in upregulation of CD39, the ectoenzyme initiating an ATP/ADP hydrolysis cascade that culminates with the generation of adenosine. Release of adenosine in the extracellular milieu leads to a decrease in cell proliferation, reduction in Th1 and Th2 development, attenuation of Th17 pathogenic potential and Treg induction.

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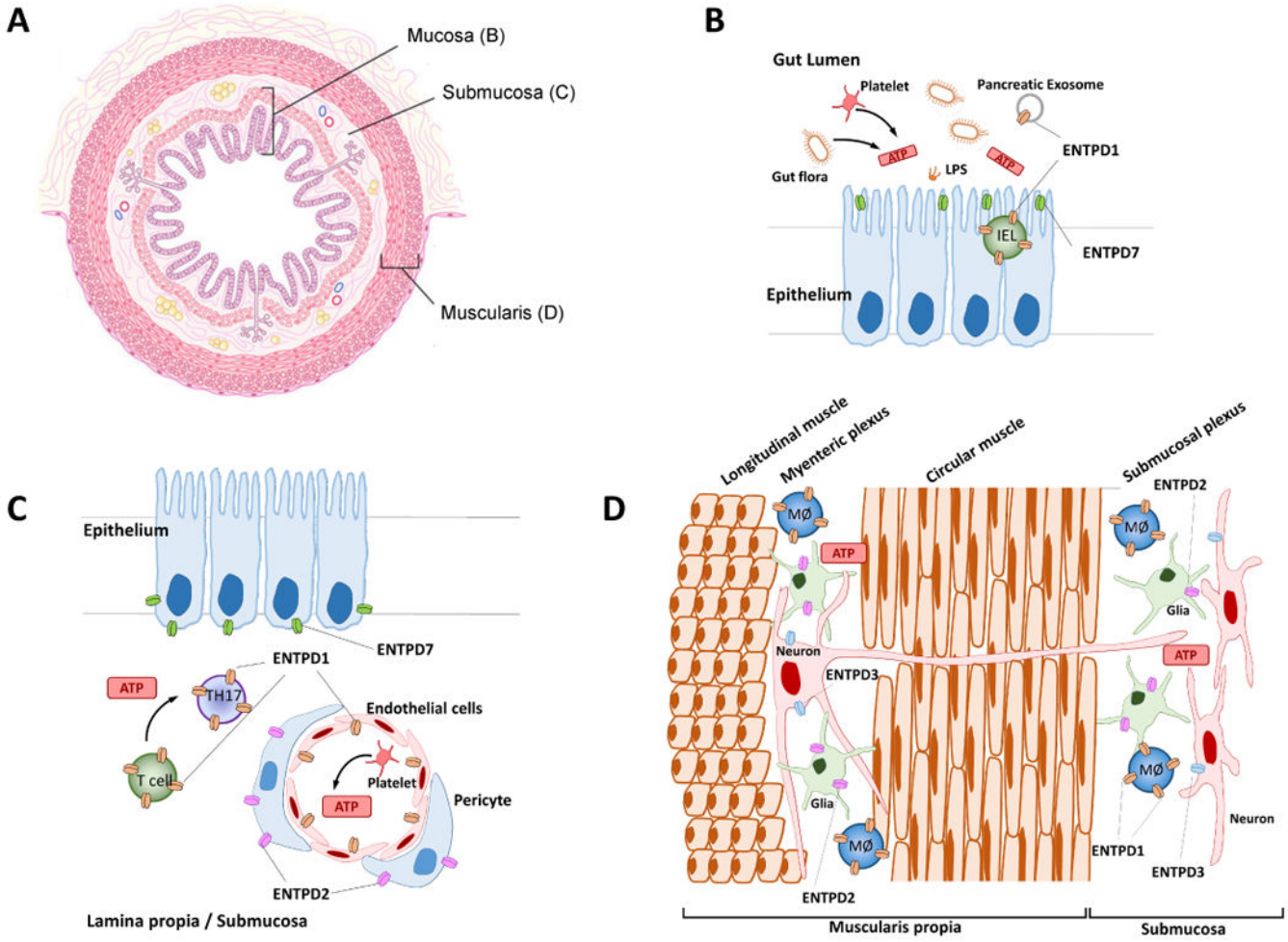

Fig. 3. Expression of select E-NTPDases in the digestive tract

A. A cross-sectional diagram of the digestive tract highlighting the three key layers. The expression of E-NTPDases in the digestive tract in relation to other cellular structures are shown in the mucosa (B), lamina propria and submucosa (C), and the muscularis (D). See text for details.