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Review

Physiological roles for ecto-5′-nucleotidase (CD73)

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Abstract

Nucleotides and nucleosides influence nearly every aspect of physiology and pathophysiology. Extracellular nucleotides are metabolized through regulated phosphohydrolysis by a series of ecto-nucleotidases. The formation of extracellular adenosine from adenosine 5′-monophosphate is accomplished primarily through ecto-5′-nucleotidase (CD73), a glycosyl phosphatidylinositol-linked membrane protein found on the surface of a variety of cell types. Recent *in vivo* studies implicating CD73 in a number of tissue protective mechanisms have provided new insight into its regulation and function and have generated considerable interest. Here, we review contributions of CD73 to cell and tissue stress responses, with a particular emphasis on physiologic responses to regulated CD73 expression and function, as well as new findings utilizing *Cd73*-deficient animals.

Introduction

Circulating or locally released nucleotides are rapidly metabolized by ecto-enzymes localized on the cell surface. Ecto-5′-nucleotidase (CD73) is a glycosyl phosphatidylinositol (GPI)-linked, membrane-bound glycoprotein which hydrolyzes extracellular nucleoside monophosphates into bioactive nucleoside intermediates [1]. Surface-bound CD73 metabolizes adenosine 5′-monophosphate (AMP) to adenosine, which when released can activate one of four types of G-protein coupled, seven transmembrane spanning adenosine receptors (AdoR) or can be internalized through dipyridamole-sensitive carriers [2]. Adenosine receptors are expressed on a wide variety of cells, and many cell types have been shown to express more than one isoform of the receptor. Likewise, activation of surface AdoR has been shown to regulate diverse physiologic endpoints. In the recent years, our understanding of nucleotide metabolic pathways has benefited from the development of genetically manipulated animals, particularly mice deficient in *Cd73* or a second nucleotide metabolizing enzyme, *Cd39* (ecto-apyrase), that catalyzes the phosphohydrolysis of ATP and ADP to AMP. Here, we build on outstanding previous reviews [1, 3] to provide concise summation of relevant studies addressing physiologic influences of CD73 in multiple settings and utilizing a variety of model systems.

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Physiological responses coordinated by CD73

Epithelial ion and fluid transport

A primary physiologic function of epithelial cells is water transport. Mucosal tissues lined by epithelia, such as the lung and intestine, accomplish this function through a coordinated series of ion transport events [4]. As part of a tissue adaptive response, a number of purine nucleotide metabolites, including adenosine, have been shown to influence epithelial electrogenic chloride secretion, the transport event responsible for mucosal hydration [4]. This aspect of epithelial function has been studied in detail utilizing models of intact epithelial cell layers coupled with electrophysiologic strategies. Original studies by Madara et al. examining biological properties of soluble mediators derived from activated inflammatory cells (e.g. neutrophils and eosinophils) identified a small, protease-resistant fraction termed neutrophil-derived secretagogue (NDS), which when incubated on epithelia, activated electrogenic chloride secretion and fluid transport. Subsequent biophysical analysis of NDS identified this molecule to be AMP [5]. With no known AMP receptor, studies turned toward defining potential metabolic pathways for adenosine generation. Biochemical and pharmacologic studies demonstrated the polarized expression of CD73 on the apical

surface of cultured and primary intestinal epithelial cells [6]. Further biochemical and morphological studies revealed that CD73 exists in both a GPI-linked surface fraction as well as in a sub-apical caveolin-rich domain within the epithelium. Such expression patterns have subsequently been shown in a variety of mucosal epithelial cell types.

At present, it is not known how CD73 directly influences ion transport properties of intact tissues. Of note on this accord, tissues with high ion transport capacities appear to express high levels of CD73. For example, as part of one study addressing the phenotype of *Cd73*$^{-/-}$ mice [7] (also see later), quantification of CD73 enzyme activity (i.e., AMP hydrolyzing activity inhibited by α,β-methylene-ADP, APCP) in a range of tissues produced several important findings. First, there was a nearly 50-fold variation in CD73 ecto-5′-nucleotidase enzyme activity in different tissues from wild-type animals. Second, of the tissues surveyed, colon showed the highest level of enzyme activity, a somewhat surprising result, as a number of previous studies suggested that the kidney likely carried the highest activity of any tissue. Instead, it was found that the colon expresses nearly twice as much activity as the kidney, with the rank order of tissue activity as follows: Colon > kidney = brain > liver > lung > heart >> muscle. Furthermore, AMP hydrolyzing enzyme activity that was not attributed to CD73 (i.e., nucleotidase and/or phosphatase activity that was not inhibited by APCP) also varied up to 20-fold between individual tissues. Why such seemingly disparate enzyme expression patterns exist is not clear at present. However, the data suggest tissue-specific patterns of extracellular nucleotide metabolism and differences in the relative contribution of CD73 to extracellular adenosine generation among individual tissues, including those important in ion transport. Taken together, these studies implicate CD73 as a metabolic control point for the activation of adenosine-mediated fluid transport that likely represents a primitive ‘flushing’ mechanism across the luminal aspect of the mucosa.

Barrier function

A number of studies have implicated CD73 in the control of tissue barrier function. Successful transmigration of leukocytes, particularly polymorphonuclear (PMN, neutrophil) leukocytes across the vascular endothelium is accomplished by temporary self-deformation with localized widening of the inter-junctional spaces [8, 9], a process with the potential to disturb endothelial and epithelial barrier function. Original studies by Lennon et al. revealed that the prominent signaling pathway for closing inter-endothelial gaps during neutrophil transmigration involved adenosine-stimulated ‘resealing’ of the barrier [10].

Until recently, only limited information existed regarding the biochemical events which regulate cellular barriers in the setting of either neutrophil activation or transmigration [8, 9]. In the course of the aforementioned studies by Lennon et al. examining interactions of leukocytes at cell–cell junctions, it was revealed that inhibition of CD73 using either APCP or anti-CD73 monoclonal antibody 1E9 inhibited the resealing of endothelial and epithelial barriers by as much as 85% [10], suggesting the necessity for extracellular nucleotide metabolism in this pathway. Subsequent studies revealed that adenosine produced from neutrophil-derived AMP was responsible for enhanced barrier function via activation of the adenosine A_{2B} receptor coupling to cytoskeletal links [11]. More recently, it was shown that in addition to (or rather than) releasing AMP, neutrophils actively release ATP following receptor-mediated stimulation [12]. Such ATP is hydrolyzed to adenosine at the endothelial cell surface through the coordinated actions of CD39 and CD73 [13]. From this perspective, some evidence exists that ecto-nucleotidases and ecto-ATPases can exist as a soluble, circulating enzymes. Yegutkin et al., for example, demonstrated that endothelial shear stress induces the release of surface protein capable of ATP and AMP phosphohydrolysis [14]. The source of this activity was the cell surface, and presumably represents soluble forms of CD73 and CD39. Likewise, this same study revealed that shear stress induces the release of endogenous ATP. It is clear exactly how neutrophils and/or endothelial cells release ATP, although several mechanisms have been proposed, including direct transport through ATP-binding cassette (ABC) proteins, transport through connexin hemichannels, as well as vesicular release [15].

Important in this regard, there may be some specificity to the regulation of barrier function by individual leukocyte populations. For example, Henttinen, et al.showed that adherent lymphocytes rapidly deaminate adenosine at the vascular interface, effectively decreasing adenosine concentrations [16]. Under such conditions, lymphocytes were shown to impair endothelial barrier function. It is possible, for example, that these differences between neutrophils and lymphocytes are explained by the relative expression patterns of adenosine deaminase, particularly given the prominent expression of lymphocyte adenosine deaminase [17].

Clearly, CD73 lies central to the regulation of tissue barriers. As an example, studies in mouse models of intestinal permeability revealed that oral delivery the CD73 inhibitor APCP increases movement of inert tracers, such as FITC-labelled dextran, across the intestinal epithelium [18]. To investigate changes in vascular permeability in *Cd73*$^{-/-}$ mice [7] (see discussion later), we have used Evan’s blue dye, which binds tightly to plasma albumin [19]. Quantification of formamide-extractable Evan’s blue from individual tissues can then be interpreted as a function of vascular leak [20]. In general, hypoxia increases vascular permeability two- to four-fold over normoxic conditions, depending on the tissue being studied [13]. Pharmacologic interventions have suggested that CD73 is protective under such circumstances, and most studies have suggested a protective role for adenosine A_2 receptors in maintaining barrier function [21]. Taken together, these studies define CD73 as a gatekeeper for the fine tuning of epithelial and endothelial permeability. Such innate protective pathways share the common strategy of increasing extracellular ade-

nosine concentrations and promoting adenosine signaling at the cell surface.

Adaptation to hypoxia

Generation of extracellular adenosine has been widely implicated as an adaptive response to hypoxia. Studies dating back more than 20 years indicated that tissue and plasma adenosine levels increase during hypoxic stress [22, 23]. For example, in human volunteers subjected to ambient hypoxia (SpO_2 = 80% over 20 min), plasma adenosine concentrations increased from 21 to 51 nM in the presence of dipyridamole, an inhibitor of adenosine reuptake [24]. Similarly, when measuring adenine nucleotide concentrations in neurally and vascularly isolated, perfused skeletal muscles of anesthetized dogs, normobaric hypoxia was associated with increases of adenosine in the venous blood, but not of AMP, ADP or ATP [25]. A possible role for adenosine during hypoxia may include vasodilation. For instance, it has been suggested that elevations in intracellular nitric oxide as a result of adenosine A_1 receptor activation may be responsible for the vasodilatory properties of adenosine [26, 27]. Thus, increases in adenosine tissue concentrations during hypoxia may promote blood flow to hypoxic tissues, thereby providing an innate protective response to hypoxia. Rather little is known about the regulation of CD73, and whether regulated expression provides a physiologic role. A number of studies have suggested that CD73 contributes to the protective aspects of adenine nucleotide metabolism during hypoxia and ischemia (see later).

Molecular mechanisms leading to elevations of adenosine in hypoxia are only now being elucidated. The cloned *Cd73* gene promoter contains a cAMP response element (CRE) [28], a consensus DNA motif which regulates transcription through the cAMP-dependent co-activator CRE-binding protein (CREB) [29]. Activation of either the adenosine A_{2A} or A_{2B} receptor elevates intracellular cAMP and CREB2, providing the possibility that the enzymatic product of CD73 (adenosine) transcriptionally regulates surface enzyme (CD73) expression. More recently, hypoxia has been shown to up regulate CD73 expression and function in several different cell types [13, 18, 30–32]. As part of these studies, we observed a rapid and prolonged induction of CD73 in epithelia [18]. Given the long lasting and robust hypoxia response observed, a candidate regulator was hypoxia-inducible factor-1 (HIF-1). As a global regulator of oxygen homeostasis, the αβ heterodimeric transcription factor HIF-1 facilitates both oxygen delivery and adaptation to oxygen deprivation [33]. HIF-1 is a member of the Per-ARNT-Sim (PAS) family of basic helix-loop-helix (bHLH) transcription factors. HIF-1 activation requires stabilization of an O_2-dependent degradation domain (ODD) in the α subunit and subsequent nuclear translocation to form a functional complex with HIF-1β and cofactors such as CBP and its ortholog p300 [34]. Under conditions of adequate oxygen supply, iron- and oxygen-dependent hydroxylation of two prolines (Pro^{564} and Pro^{402}) within the ODD of HIF-1α initiates the association with the von Hippel–Lindau tumor suppressor protein (pVHL) and rapid degradation via ubiquitin-E3 ligase proteasomal targeting [35, 36]. A second hypoxic switch operates in the carboxy terminal transactivation domain of HIF-1α. Here, hypoxia blocks the hydroxylation of Asp^{803}, thereby facilitating the recruitment of CBP/p300 [37].

A search of the cloned *Cd73* gene promoter revealed a classic HIF-1 binding DNA consensus motif 5′-CCGTG-3′ located at positions −367 to −371 relative to the major transcription start site [18]. However, the existence of a HIF-1 binding consensus site is not evidence for a HIF-1-mediated response. A classic HIF-1 response element (HRE) is defined as a *cis*-acting transcriptional regulatory sequence located within 5′-flanking, 3′-flanking or intervening sequences of target genes. Three approaches were used to define a role for HIF-1 in the induction of CD73 expression. First, HIF-1α antisense oligonucleotides significantly reduced the induction of CD73 under hypoxic conditions in bovine aortic endothelial cells transiently transfected with a *Cd73* promoter luciferase reporter construct. Second, truncated reporter constructs identified a functional hypoxia responsive element located between −518 and −92 nucleotides upstream of the major transcription start site of the *Cd73* gene. Finally, a two nucleotide mutation in a consensus HIF-1α binding site in the *Cd73* promoter led to a complete loss of hypoxia inducibility of the luciferase reporter construct. These studies also implicated transcriptional repressor elements for CD73 expression in hypoxia. For example, some of the larger truncated promoter constructs showed increased hypoxia-inducibility than the full length promoter, suggesting the presence of at least partial repressor activity [18]. A number of consensus binding sites map to this region of the promoter, and while we have not directly addressed this issue, at least two transcription factors (GATA-1 and GATA-2) have recently been implicated in gene repression in hypoxia. Thus, it is probable that both positive and negative regulatory pathways contribute to molecular regulation *Cd73* promoter activity.

Ischemic preconditioning

Cells of the cardiovascular system generate and release adenosine in increasing quantities during stress or when subjected to injurious stimuli. This increased adenosine can interact with surface receptors in myocardial, vascular, fibroblast, and inflammatory cells to modulate cellular function and phenotype [13]. Via these receptor-dependent and independent (metabolic) pathways, adenosine can substantially modify the acute response to ischemic insult, in addition to generating a more sustained ischemia-tolerant phenotype (preconditioning). However, the molecular basis for acute adenosinergic cardioprotection remains poorly understood [38].

The potential for adenosine to exert cardioprotective influences on the ischemic myocardium is well-documented [38]. While the source of interstitial adenosine in hypoxic tissue has been the basis of much debate, it is generally

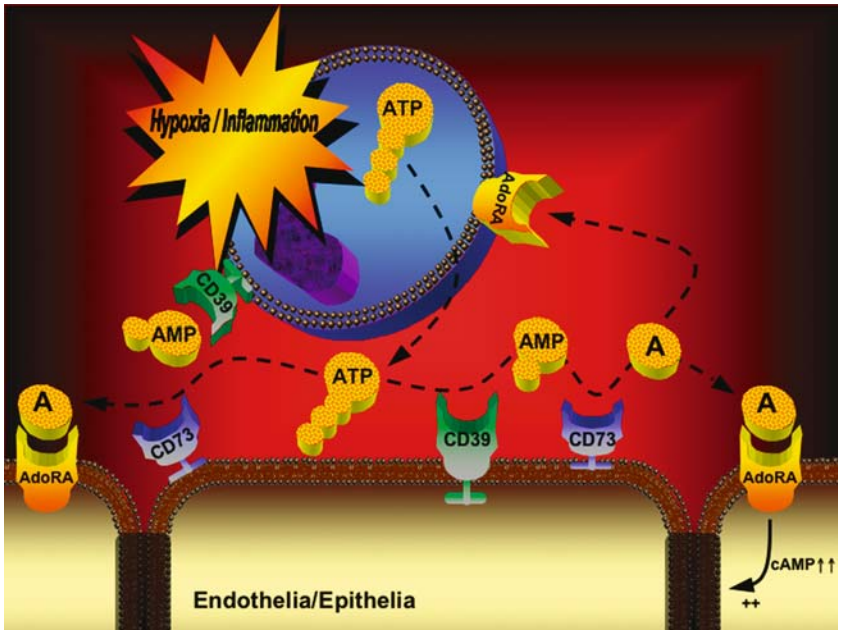

Figure 1. Model of coordinated nucleotide metabolism and nucleoside signaling in hypoxia and inflammation. In areas of ongoing inflammation, diminished oxygen supply coordinates the induction of CD39, CD73, and the adenosine A_{2B} receptor. At such sites, activated neutrophils provide a readily available extracellular source of ATP, that through two enzymatic steps is converted to extracellular adenosine. Activation of surface adenosine receptors promotes a variety of protective physiologic responses (see text). Abbreviations: *A*, adenosine; *AdoRA* A_2, adenosine receptor; *AMP*, adenosine monophosphate; *ATP*, adenosine triphosphate; *cAMP*, cyclic AMP.

accepted that the dephosphorylation of AMP by CD73 represents the major pathway of extracellular adenosine formation during oxygen supply imbalances [2]. Extracellular adenosine production in the ischemic myocardium, for example, is attributable to activity of CD73 [39], and both CD73 activity and adenosine metabolism have been demonstrated in cardiac pre-conditioning by brief periods of ischemia [40, 41]. Increased CD73 activity in ischemic preconditioning (IP) has been attributed to a variety of acute activation pathways [42], and as alluded to above, CD73 is transcriptionally regulated by HIF-118. Because ecto-5′-nucleotidase (CD73) is induced during ischemia and hypoxia [13], it is thought to be primarily responsible for adenosine production under those circumstances.

Some evidence indicates that the increase in CD73 in myocardium subjected to IP may originate from myocytes, but the immediate influence of myocytes on CD73 activity is not well understood [38]. The relative importance of CD73 in cardiac tissue was recently demonstrated using *Cd73*$^{-/-}$ mice [43]. In the isolated perfused heart, it was shown that AMP hydrolyzing activity attributable to CD73 accounted for 60% of the total hydrolytic activity during a single passage through the coronary vasculature. Furthermore, histochemical analysis revealed CD73 to be the predominant AMPase associated with the vascular endothelium of large conduit vessels such as the aorta, carotid, and coronary artery with no measurable contribution by alkaline phosphatase [43].

Activation of adenosine A_1 and possibly A_3 receptors is believed to protect heart and other tissues by preconditioning through a pathway including protein kinase C and mitochondrial K_{ATP} channels. Activation of adenosine A_{2A} receptors seems to limit reperfusion injury by inhibiting inflammatory processes in neutrophils, platelets, macrophages and T cells [2]. The role of the adenosine A_{2B} receptor in IP is unknown, although some evidence suggests that it may be involved in the inhibition of cardiac fibroblast proliferation, protein, and collagen synthesis [44]. It is worth noting here that much of the controversy regarding receptor identities stems from the major problem that many receptor agonists and antagonists previously thought to be highly selective are in fact capable of interacting with multiple receptor subtypes at concentrations typically employed [38].

CD73 and inflammation

For more than two decades, it has been appreciated that adenosine attenuates potentially harmful aspects of neutrophil activation [45]. More recent studies have focused on targeting adenosine receptors to limit tissue injury in a variety of diseases using either native adenosine or pharmacological agonism/antagonism with receptor-selective analogs [2, 46, 47]. Less is known about the influence of endogenously generated adenosine on neutrophil function. Original work by Rosengren, et al. [48] using the non-selective adenosine receptor antagonist 8-phenyl-theophylline demonstrated enhanced inflammation in the hamster cheek pouch, thereby suggesting tonic regulation of neutrophil receptors to endogenous adenosine sources. More recent studies using new reagents and new insight into metabolic pathways to generate extracellular adenosine (i.e., CD39 and CD73) provide an opportunity to understand these pathways with more clarity.

In other examples, acute hypoxemia has been shown to enhance neutrophil activation responses [49]. For instance,

during episodes of hypoxia, neutrophils are mobilized from the intravascular space to the interstitium, and such responses may contribute significantly to tissue damage during consequent reperfusion injury [50–52]. Moreover, emigration of PMN through the endothelium and epithelium may lead to a disruption of tissue barriers [53] and create the potential for extravascular fluid leakage and subsequent edema [54, 55].

By contrast, transcriptional pathways mediated by HIF-1 may serve as barrier-protective elements during inflammatory hypoxia. For example, experimental studies of murine colitis have revealed extensive mucosal hypoxia and concomitant HIF-1 activation [56]. Mice engineered to conditionally delete intestinal epithelial HIF-1α exhibit more severe clinical symptoms of colitis, while conditional increases in epithelial HIF-1 are protective. Furthermore, colons with constitutive activation of HIF-1 displayed increased expression levels of HIF-1 regulated barrier-protective genes (multidrug resistance gene-1, intestinal trefoil factor, CD73), attenuating the loss of barrier function during colitis *in vivo*. CD73 was particularly interesting from this perspective. During active phases of colitis, CD73 mRNA was increased ~4 fold in wild-type animals. Parallel analyses in animals expressing consititutively active HIF-1 revealed a nearly 18-fold increase in CD73 mRNA [56]. Such findings confirm previous observations of HIF-1 dependent regulation of CD73 expression, and define an inflammatory metabolic loop involving a contribution of hypoxia, a condition we have termed 'inflammatory hypoxia' [57].

The vascular endothelium is the primary interface between tissue inflammatory signals and circulating leukocytes [58]. As such, the endothelium is central to the orchestration of leukocyte trafficking in response to chemotactic stimuli. This critical anatomic location places vascular endothelial cells in an ideal position to coordinate extracellular metabolic events important to endogenous anti-inflammatory responses. We have recently identified a neutrophil-endothelial cell crosstalk pathway that is coordinated by hypoxia (see Figure 1). This pathway utilizes extracellular nucleotide substrates liberated from different cell types. Extracellular ATP release has been shown from endothelial cells, particularly under sheer conditions or in hypoxia. In addition, neutrophils can release ATP in an activation-dependent manner [12, 13]. Recent studies from our lab indicate that neutrophils express CD39, and can therefore also contribute to the extracellular metabolism of ATP (manuscript in preparation).

Activated platelets comprise an additional source of extracellular adenine nucleotides [59, 60]. The generation of AMP from ATP/ADP by endothelial CD39 has been viewed as a protective, thromboregulatory mechanism for limiting the size of the hemostatic plug [60, 61]. Metabolism of adenine nucleotides derived from activated platelets is crucial in limiting excessive platelet aggregation and thrombus formation [62, 63]. Similarly, excessive platelet accumulation and recruitment can be treated with soluble forms of CD39 [64, 65]. Moreover, a thromboregulatory role was demonstrated in a model of stroke, where *Cd39*-null mice showed increased infarction size that was reduced by treatment with soluble CD39 [66]. Surprisingly, targeted disruption of *Cd39* resulted in prolonged bleeding and increased vascular leak and fibrin deposition in hypoxemia [67], suggesting a dual role for ATP metabolism by CD39 in modulating both hemostatic and thrombotic reactions. This latter observation may be related to an activation and desensitization of the purinergic type $P2Y_1$ receptor by ATP. Engagement of the $P2Y_1$ platelet receptor appears to be crucial to the process of platelet activation. As such, *$P2Y_1$*-deficient mice exhibit signs of prolonged bleeding time and resistance to thromboembolism [68]. In contrast to these studies, we observed a barrier-protective influence of CD39 during hypoxia that was not related to the activation of P2 receptors, but rather to the downstream metabolism and signaling of ATP metabolites (especially adenosine).

Extracellular ATP is readily converted to adenosine on the endothelial surface, due to the sequential enzymatic function of CD39 and CD73. Adenosine binds to surface expressed adenosine receptors on PMN to limit excessive accumulation of PMN within tissues, functioning as a feedback loop to attenuate potential tissue injury [13]. With regard to this latter point, it was recently shown that hypoxia coordinates both transcriptional and metabolic control of the surface ecto-nucleotidases CD39 and CD73 [13, 18, 32], and as such, significantly amplifies the extracellular production of adenosine from adenine nucleotide precursors. In fact when using *Cd39*- and *Cd73*-null animals, we found that extracellular adenosine produced through adenine nucleotide metabolism during hypoxia is a potent anti-inflammatory signal for PMN *in vivo*. These findings identify CD39 and CD73 as critical control points for endogenous adenosine generation and implicate this pathway as an innate mechanism to attenuate excessive tissue PMN accumulation [13].

Host–microbial interactions

It is recently been appreciated that CD73 may also contribute in host responses to microbial infection. While these studies are clearly in their infancy, this area is likely to prove fruitful with further investigation. For example, it is now appreciated that a symptomology associated with some intestinal pathogens may be related to extracellular metabolism of nucleotides. Indeed, Crane et al. showed that damage related to epithelial infection by enterpathogenic *E. coli* liberates extracellular ATP, and via CD73-dependent pathways generates adenosine [69]. As alluded to above, adenosine is a potent secretagogue, and under such circumstances, may promote symptoms of secretory diarrhea in the intestine.

Likewise, some evidence suggests that CD73 may contribute to microbial responses mediated by Toll-like receptors (TLRs), although most studies have been indirect. TLR signaling pathways are currently an area of intense investigation, and while many cell types express machinery for TLR-mediated signaling, the most thoroughly studied have been those of monocytes/macrophages as well as

dendritic cells. In this regard, dendritic cells of various lineages appear to express CD73, and it has been suggested that such expression may regulate diverse functions ranging from angiogenesis to B-cell maturation [70, 71]. Frantz et al., have suggested that signaling through TLR and adenosine (via stress activated CD73) may provide a contextual 'injury' signal, and among other signaling pathways, may promote a strong angiogenic response [72]. In strong agreement, work by Pinhal-Enfield et al. and Leibovich et al. defined synergy between various TLR agonists and adenosine receptors with regard to angiogenesis [73, 74]. Under these circumstances, agonists of TLR 2, 3, 4, 7 and 9 synergized with A_2 receptors to induce vascular endothelial growth factor and repress tumor necrosis factor. The exact contribution of CD73 to these models is unclear, but better defining the source(s) of endogenous adenosine should shed light on these interesting pathways.

CD73 may also contribute to responses following virus infection. For example, Kas-Deelen et al. recently reported that endothelial infection by cytomegalovirus (CMV) increases expression and activity of both ecto-ATPase(s) and CD73 [75]. These findings were based on studies which indicated that CMV-infected endothelia have increased turnover of 5′-AMP, and that soluble mediators derived from CMV-infected cultures attenuate neutrophil activation responses. Systematic characterization of this model identified the coordinated extracellular metabolism of ATP and AMP to adenosine as the functional link. While it is unclear how CMV enhances ecto-nucleotidase activity and/or expression, it is possible that viral infection could provide a feed-forward host response to promote CD73 expression and activity. For example, Niemela has shown that cytokines induced by virally infected cells (e.g., interferon-alpha) prominently induce CD73 expression and adenosine production on the endothelium *in vitro* and *in vivo* [76]. Such responses were, at least in part, cell-type specific for endothelial cells, and were not observed in lymphocytes. These findings provide important insight into mechanisms of CD73 regulation, and further define the importance of CD73 in innate immune responses.

Studies in *Cd73*-deficient mice

At least three separate groups have generated *Cd73*-deficient mice within the course of a two-year span [7, 43, 77], each targeting one or more exons required for the ecto-5′-nucleotidase enzyme activity of CD73. *Cd73*-deficient mice have no obvious phenotype when maintained in conventional housing. They breed normally and have average sized litters. $Cd73^{+/+}$, $Cd73^{+/-}$, and $Cd73^{-/-}$ pups are born to heterozygous parents in the expected 1:2:1 ratio. Below are some characteristics of physiologic response observed in these animals.

Renal function

It has long been known that adenosine is an important mediator in the kidney, primarily through regulation of glomerular filtration. Tubuloglomerular feedback involves a functional interplay between the tubular epithelium in the environment of the macula densa and underlying arteriole smooth muscle cells. As salt concentrations increase in the luminal fluid surrounding the macula densa, smooth muscle activation results in vasoconstriction of the arterioles. As a result, both the glomerular filtration pressure and filtration rates decrease. To assess the contribution of CD73-generated adenosine in local hemodynamic control mechanisms in the kidney, Castrop et al. compared tubuloglomerular feedback in the kidneys of $Cd73^{-/-}$ and wild type mice [77]. Interestingly, kidney function of *Cd73*-deficienct mice was found to be normal with respect to renal blood flow, renal vascular resistance, stimulation of renin secretion by furosemide, plasma osmolarity, and plasma concentrations of Na^+, Cl^-, BUN, creatinine, uric acid, and total protein. However, in response to saturating increases in tubular perfusion flow, $Cd73^{-/-}$ animals demonstrated significantly decreased reductions in stop flow pressure and superficial nephron glomerular filtration rates compared to wild type animals. Furthermore, although wild type mice showed relatively constant tubuloglomerular feedback responses during prolonged perfusion of the loop of Henle, the residual feedback response was nearly lost in $Cd73^{-/-}$ mice. Nevertheless, isolated afferent arterioles from $Cd73^{-/-}$ mice showed normal contractile responses to exogenous adenosine, suggesting that the observed deficiencies in tubuloglomerular feedback responses were due to decreased concentrations of extracellular adenosine, rather than any defects in adenosine receptor activation. In total, it was concluded that CD73 serves as an important means of communication between the macula densa and the underlying smooth muscle cells.

Hypoxia and vascular leak

Progress has been made in defining the role of CD73 in hypoxia adaptation *in vivo* using *Cd73*-deficient mice [7]. These mice have provided some insight into adaptative hypoxia responses, and strongly extend the previous molecular studies of HIF-1α-dependent regulation of CD73 expression. For example, as discussed above, Thompson, et al. have recently shown that vascular leak syndromes associated with hypoxia are significantly accentuated in mice lacking CD737. In an attempt to define the role of CD73 in vascular permeability, we used this hypoxia model and compared wild-type and $Cd73^{-/-}$ mice administered either vehicle or the 5′-nucleotidase inhibitor APCP. These studies revealed a profound increase of hypoxia-induced vascular leak in different organs (lung, heart, intestine, kidneys) in response to CD73 inhibition or genetic deficency. Pulmonary leak was particularly obvious in these mice. Indeed, lung vascular leak was highly influenced by exogenous administration of APCP in wild-type animals, and the vascular leak phenotype was most prominent in the lungs of $Cd73^{-/-}$ mice. Closer histological examination revealed perivascular interstitial edema with inflammatory infiltrates surrounding the larger con-

ducting vessels of the pulmonary vasculature in *Cd73*$^{-/-}$ animals subjected to hypoxia. Vascular leak was confirmed by assessment of lung wet:dry ratios, with a nearly 70% increase in lung water content of *Cd73*$^{-/-}$ compared to wild type mice.

These mice have also been useful in undersanding the basic aspects of vascular permeability in intact organs. Our studies have consistenty demonstrated that adenosine promotes vascular barrier, and since elevation of cAMP is associated with increased barrier function [78], it is likely that our results represent activation of adenosine A_{2A} and/or A_{2B} receptors. The A_{2B} receptor may well be the target, as A_{2B} receptor mRNA is up regulated by hypoxia and A_{2B} receptor antagonists neutralize ATP-mediated changes in post-hypoxic endothelial barrier function [13]. Additionally, experiments with the adenosine receptor antagonists MRS 1754 and ZM241385, suggest that both the A_{2A} and A_{2B} receptors contribute to the changes in vascular barrier during hypoxia [7]. An A_{2B} receptor gene-targeted mouse will be required to confirm this conclusion with certainty. Alternatively, inosine could mediate these responses. We think this is unlikely since inosine binds primarily to the A_3 receptor [79] (a class of adenosine receptor that mediates decreased cAMP), and it is likely that *Cd73*$^{-/-}$ mice retain the ability to generate inosine through the combined action of AMP deaminase and cytoplasmic nucleotidases. The use of animals with constitutive gene deletions provides a global view of gene function, but fails to reveal how local changes may impact a given response. Future studies using animals with conditionally deleted *Cd73* will likely provide additional insight into these issues.

Platelet function

Nucleotides and nucleotide metabolism have been widely implicated in platelet function [80, 81]. *Cd73*-deficient animals have revealed some insight with regard to the role of CD73-generated adenosine in platelet thrombosis *in vivo* [43]. Initial studies of ADP-stimulated platelet aggregation *ex vivo* have not revealed significant differences between wild-type and *Cd73*-deficient animals, suggesting that platelet function is intrinsically normal in *Cd73* gene-targeted mice. However, bleeding time after tail tip resection and vessel occlusion induced by free radical injury were significantly reduced in the *Cd73*-deficient animals, suggesting a degree of platelet dysfunction. Other studies have indicated that platelet cAMP is reduced in *Cd73*-deficient mice, suggesting that plasma adenosine levels regulate basal platelet cAMP, and that decreases in circulating adenosine in *Cd73*-deficient animals contribute to such changes. Additional studies of platelet function and clotting will be necessary to define the contribution of CD73 to these pathways.

Therapeutic potential for CD73

The design and implementation of adenosine receptor agonists and antagonists is currently an area of intense investigation [80, 81]. While drugs targeting adenosine receptors hold great promise in a variety of diseases, specificity and pharmacodynamics have been significant challenges [2]. As an alternative to modulating adenosine receptor signaling with agonists or antagonists, it may be possible to manipulate extracellular adenosine concentrations. Unfortunately, under many circumstances the source of endogenous adenosine is unclear. However, in cases where the prominent pathway involves extracellular metabolism of ATP and/or AMP, it may be possible to regulate these metabolic steps.

One strategy may be to enhance the extracellular adenosine formation. Some work has been done in this regard. For example, methotrexate and sulfasalazine have been shown to increase extracellular adenosine [82]. Such increases in adenosine were shown to involve a CD73-mediated step both *in vitro* and *in vivo*. Alternatively, targeted use of soluble nucleotidases could provide an additional mechanism. Administration of 5′-nucleotidase has been shown to be beneficial in a number of experimental scenarios. For example, soluble 5′-nucleotidase promotes vascular barrier function and decreases neutrophil accumulation in inflammatory models [7, 12]. A significant limitation for this line of work has been the identification of a reliable source of purified protein. Ironically, one of the major sources of 5′-nucleotidase may be snake venoms, particularly those from rattlesnakes (genera *Crotalus* and others) [83]. It is thought that snakes utilize purines as an effective, multifunctional means of envenomation [84].

CD73 directed therapies have not been well-developed. In our own experience, we have documented use of the CD73 inhibitor αβ-methylene ADP (APCP) in various murine models [7, 12, 18]. APCP is well tolerated, biologically available through the oral route, and non-toxic in mice up to 60 mg/kg/day. Anecdotally, we have noticed that APCP treatment of mice appears to promote subtle increases in aggressive behavior and anxiety (unpublished observation). It is possible that this behavior is related to previous findings of increased aggression in adenosine A_1r- and $A_{2A}r$-deficient mice [85, 86], and may suggest that APCP is biologically available across the central nervous system. While many studies have suggested that adenosine is beneficial for most host responses, there may be examples where inhibition of adenosine generation (e.g., by inhibition of CD73) is warranted. As alluded to above, during enteric pathogen infection, adenosine may promote water transport across intestinal epithelia and the symptoms of secretory diarrhea. For these purposes, CD73 inhibitors such as APCP could prove effective as anti-diarrheals. In this same context, it is possible that pulmonary edema related to infectious pneumonia or inflammation may benefit from the use of CD73 inhibitors. Studies directed at defining these principles are currently underway.

Given the established association between angiogenesis and adenosine A_2 receptor activation, the controlled regulation of CD73 activity could influence angiogenesis. It is possible, for example, that systemic administration

and/or targeting of CD73 inhibitors could prove beneficial for inhibition of tumor angiogenesis, currently an area of keen interest in cancer research. This is particularly compelling given the known association between hypoxia and the tumor microenvironment [87], wherein hypoxia and HIF-1 activation are potent transcriptional stimuli for CD73 expression [18] (also see above). Under such circumstances, it is reasonable that many tumors might over-express CD73. Thus, inhibition of adenosine production by CD73 could be a therapeutic target for the prevention of tumor angiogenesis and metastasis. Experiments to test this hypothesis are in progress.

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