CELL TYPE AND TISSUE SPECIFIC FUNCTIONS OF CD73

Marquet Minor

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Approved by:

A. Sidney Barritt

Temitope Keku

Lori O'Brien

Scott Randell

Natasha Snider

ABSTRACT

Marquet Minor: Cell Type and Tissue Specific Functions of CD73
(Under the direction of Natasha Snider)

CD73 is a ubiquitously expressed glycosylphosphatidylinositol (GPI)-anchored glycoprotein that converts extracellular adenosine 5'-monophosphate (AMP) to adenosine. CD73 couples closely with ecto-apyrase (CD39), which supplies the AMP substrate via sequential dephosphorylation of extracellular ATP. CD73-generated adenosine functions in an autocrine and paracrine manner to control numerous physiological responses by activating one of four subtypes of G-protein-coupled adenosine receptors: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. Missense mutations in the CD73-encoding gene *NT5E* cause the rare, adult-onset vascular disease named 'arterial calcifications due to deficiency of CD73' (ACDC). Aside from direct human disease involvement, cellular and animal model studies have revealed key functions of CD73 in tissue homeostasis and pathophysiologic responses in the cardiovascular and central nervous system, as well as epithelial tissues, including the lung, kidney and liver. This review covers CD73 functions in multiple organ systems, with a focus on novel findings from the last 3-5 years.

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LIST OF ABBREVIATIONS AND SYMBOLS

Γδ	Gamma-delta
A_1R	Adenosine receptor A_1
$A_{2A}R$	Adenosine receptor A_{2A}
$A_{2B}R$	Adenosine receptor A_{2B}
A_3R	Adenosine receptor A_3
A_1R-/-	Adenosine receptor 1 knockout mouse model
$A_{2A}R$-/-	Adenosine receptor 2A knockout mouse model
$A_{2B}R$-/-	Adenosine receptor 2B knockout mouse model
ACDC	Arterial calcifications due to deficiency of CD73
AMP	Adenosine 5'-monophosphate
AMPASE	Class of enzymes that catalyze the dephosphorylation of AMP into a nucleoside and a free phosphate ion
APCP	Adenosine 5′-(α,β-methylene)diphosphate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
CAMP	Cyclic adenosine monophosphate
CCl4	Carbon tetrachloride

CD39	Ecto-apyrase
CD4+	Cluster of differentiation 4 expressing
CD73	Ecto-5’-nucleotidase
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis
ELF2	E74-like factor 2
ENT	Equilibrative nucleoside transporter
EPDC	Epicardium-derived cell
FP-1201	Recombinant human interferon beta-1a
GPI	Glycosylphosphatidylinositol
GVHD	Graft vs host disease
IB4	Isolectin B4
IFN-β1	Interferon beta
IFN-γ	Interferon gamma
IGG3	Immunoglobulin G 3
IGM	Immunoglobulin M

IL1-β	Interleukin 1 beta
IL-6	Interleukin 6
IL-11	Interleukin 11
IL17A	Interleukin-17A
IL-17	Interleukin 17
I/R	Ischemia-reperfusion
K8/K18	Keratins 8 and 18
L3-5	Lumbar vertebrae 3-5
LPS	Lipopolysaccharide
M1	Pro-inflammatory macrophages
M2	Macrophages that are anti-inflammatory
MC3T3-E1	Mouse osteoblastic cell line
MDB	Mallory-Denk bodies
MI	Myocardial infarction
MRGPRD	Mas-related G-protein coupled receptor member D
MSC	Mesenchymal stem cell
NAD	Nicotinamide adenine dinucleotide
NECA	5′-(N-ethylcarboxamido)adenosine

NT5E	Ecto-5’-nucleotidase-encoding gene
NT5E-/-	NT5E knockout mouse model
P62	Nucleoporin 62
PAP	Prostatic acid phosphatase
PH	Measure used to determine the acidity or basicity of a solution
SP1	Specificity protein 1
TAA	Thioacetamide
TGFβ	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1
TH1	Type 1 helper T cells
TH17	Pro-inflammatory helper T-cells that produce interleukin 17
TNAP	Tissue Non-specific Alkaline Phosphatase
TNF-α	Tumor necrosis factor alpha
TRPV1	Transient receptor potential cation channel subfamily V member 1
VEGF	Vascular endothelial growth factor
WT	Wild-type

CHAPTER 1: INTRODUCTION

The enzymatic dephosphorylation of 5´-nucleotides, such as adenosine 5´-monophosphate (AMP) is widespread across mammalian systems and represents a key step in purine salvage pathways to modulate purinergic signaling (Ipata & Balestri, 2013; Zimmermann, Zebisch, & Strater, 2012). This catalysis occurs inside the cell or in the extracellular space. The major enzyme catalyzing the formation of extracellular adenosine from AMP is ecto-5´-nucleotidase, encoded by the *NT5E* gene (Zimmermann et al., 2012). Ecto-5'-nucleotidase was named CD73 in 1989 after it was shown that its engagement by specific antibodies induced T cell activation (Thompson, Ruedi, Glass, Low, & Lucas, 1989). In this review, we refer to the protein as CD73 and the gene as *NT5E*. Extracellular adenosine produced by CD73 acts on adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R) to activate downstream signaling (Antonioli, Blandizzi, Pacher, & Hasko, 2013; Chen, Eltzschig, & Fredholm, 2013; Eltzschig, 2009; Fredholm, 2007). Through its function as the major extracellular source of adenosine, CD73 is a key regulator of tissue homeostasis and pathophysiologic responses related to immunity, inflammation, and cancer, covered in recent reviews (Antonioli, Pacher, Vizi, & Hasko, 2013; Beavis, Stagg, Darcy, & Smyth, 2012; Colgan, Eltzschig, Eckle, & Thompson, 2006; Roberts, Lu, Rajakumar, Cowan, & Dwyer, 2013). The focus of this review is to highlight known and emerging tissue-specific functions of CD73 in the brain and spinal cord, the heart, and epithelial tissues.

Structural and molecular features of CD73. Molecular and structural studies have revealed that CD73 is glycosylphosphatidylinositol (GPI)-anchored protein that is functionally coupled with another enzyme, ecto-apyrase (CD39), which supplies the AMP substrate for CD73(Antonioli, Pacher, et al., 2013). Characterization of the human CD73 protein using X-ray crystallography has revealed that human CD73 is a dimer and that its dimerization interface is formed via the C-terminal domain (K. Knapp et al., 2012). The crystal structure of CD73 from the *T. thermophiles* bacteria species differs from the human structure at the N-terminal metal ion-binding domain and the C-terminal substrate-binding domain. While structurally the bacterial and human proteins are different, the *ecto*-nucleotidase function is evolutionarily conserved (Knapp, Zebisch, & Strater, 2012).

Evidence for non-enzymatic CD73 functions. CD73 function and regulation differ between cell types with respect to phospholipase sensitivity, shedding from the cell membrane, and ability to trigger intracellular signals in response to antibody stimulation, which suggests a potential signaling function independent of adenosine (Airas et al., 1997). Indeed, several studies highlight important functions of CD73 that are independent of its activity as an AMPase, including: (i) T-cell activation via protein interactions to deliver a co-stimulatory signal (Resta & Thompson, 1997); (ii) promoting adhesion of lymphocytes to the endothelium (Airas et al., 1995) by inducing integrin clustering; (Airas, Niemela, & Jalkanen, 2000) (iii) conferring resistance to apoptosis of leukemic cells via GPI-anchor-dependent mechanisms (Mikhailov et al., 2008); (iv) inducing phosphorylation of endothelial and lymphocyte proteins in response to antibody ligation (Airas et al., 1997; Dianzani et al., 1993); and inhibition metastasis of breast cancer cells after membrane clustering and internalization (Terp et al., 2013). Presently it is not

known whether these observations are linked to a common function, such as CD73 potentially functioning as a receptor for a putative ligand, which has yet to be defined.

Physiological and disease relevance of CD73. CD73 is ubiquitously expressed in humans, but its expression pattern varies across tissues and cells types (Figure 1). CD73-generated adenosine plays a pivotal role in several physiological functions including: epithelial ion and fluid transport, tissue barrier maintenance, hypoxia, ischemic preconditioning, and inflammation (Colgan et al., 2006). Mutations in *NT5E* leading to catalytically nonfunctional CD73 cause ACDC, a disease that manifests with symptomatic arterial and joint calcifications in humans (St Hilaire et al., 2011). ACDC has an autosomal recessive pattern of inheritance and is adult onset.

The mouse *Nt5e-/-* knockout model recapitulates some, but not the full spectrum, of the human pathology (Li, Price, Sundberg, & Uitto, 2014). Part of the mechanism by which mutations of the human *NT5E* gene contribute to disease is attributed in part to defective intracellular trafficking of CD73 (Fausther, Lavoie, Goree, Baldini, & Dranoff, 2014). Global deletion of the mouse gene (*Nt5e*) leads to hypoxia-induced vascular leakage in multiple tissues, most profoundly in the lung (Thompson et al., 2004). The *Nt5e*$^{-/-}$ mice, which were generated and first described by Linda Thompson and her colleagues fifteen years ago (Thompson et al., 2004) have been used in numerous studies since then to uncover a number of phenotypes, as described in Figure 2. The initial characterization of the *Nt5e*$^{-/-}$ mice provided the foundational knowledge to further examine CD73 in various pathophysiological states. This review will address the current understanding of CD73 function and regulation landscape at the cell and tissue-specific level.

CHAPTER 2: CD73 FUNCTIONS IN THE CARDIOVASCULAR SYSTEM

CD73 expression and distribution in the cardiovascular system. Robert Berne showed 55 years ago that adenosine production in the heart is stimulated in response to hypoxia and proposed that adenosine mediates local metabolic control of coronary blood flow (*the adenosine hypothesis*) (Berne, 1963). A brief historical account of some of the studies (Eltzschig et al., 2003; Koszalka et al., 2004) leading to the recognition of CD73 as a key player in the cardiovascular system was provided by Ray Olsson in 2004 (Olsson, 2004). Other relevant work on CD73 and purinergic signaling in the heart was highlighted in a review by Burnstock and Pelleg (Burnstock & Pelleg, 2015). Here we focus on the most recent work regarding cell type specific functions of CD73 in the cardiovascular system.

In the cardiovascular system CD73 is expressed on smooth muscle cells (Yang et al., 2015), endothelial cells (M. Ohta et al., 2013; Pluskota et al., 2013), and resident lymphocytes (Bonner, Borg, Burghoff, & Schrader, 2012). The Human Protein Atlas reports moderate levels of CD73 protein expression on normal human smooth muscle cells, endothelial cells, and cardiomyocytes (Uhlen et al., 2015), whereas Bonner et al. noted lack of CD73 on these cell types in mouse heart (Bonner et al., 2013). It is possible that disruption of the tissue architecture during digestion and processing for cell sorting activated mechanical signaling to trigger downregulation of surface CD73 from non-immune cells in the latter study (Bonner et al., 2013). For example, hepatocytes *in vivo* express CD73, but this expression is dramatically downregulated during the process of liver digestion and cell isolation (Snider et al., 2013). One

potential molecular mechanism may be through the actions of the mechanosensitive cytoskeletal protein kindlin-2, a modulator of integrin signaling in endothelial cells and cardiomyocytes (Dowling et al., 2008) that regulates CD73 trafficking to the membrane (Pluskota et al., 2013). In the Bonner et al. study, the resident leukocyte population, in particular T cells had the highest level of CD73 per cell and highest proportion of CD73-positive cells. However, ~50% of the resident T cells expressed CD73, in contrast to almost ~90% of T cells in the circulation that were CD73 positive. Therefore, assessing functional contributions to CD73 on different cell types in the heart and vasculature will require additional studies utilizing cell type-specific deletion of CD73 in cells and in mice. The key functions of CD73 reported relate to supporting recovery after myocardial infarction (MI), protection during in heat failure (Quast, Alter, Ding, Borg, & Schrader, 2017), promoting atherosclerosis (Yang et al., 2015), supporting aortic valve function (Zukowska et al., 2017) and blocking arteriogenesis (Boring et al., 2013). We will highlight recent work related to the cell-specific roles of CD73 in MI below.

Cell type-dependent roles of CD73 after myocardial infarction (MI). Healing and recovery of tissue function following myocardial infarction is dependent on T cell-expressed CD73, which decreases inflammation through the generation of adenosine (Borg et al., 2017). Specifically, circulating T cells invade the injured heart after infarction and upregulate expression of hydrolyzing enzymes that act on ATP, cAMP, and NAD, culminating in adenosine production via CD73. Activation of $A_{2A}R$ and $A_{2B}R$ leads to reduction in the release of inflammatory mediators. T cells from *Nt5e*$^{-/-}$ mice are skewed toward Th1 and Th17 types, resulting in increased levels of their respective pro-inflammatory cytokine products IFN-γ and IL-17 (Borg et al., 2017). When co-cultured with mesenchymal stem cells, monocytes upregulate CD73 expression in vitro and in vivo in post-MI swine heart (Monguio-Tortajada et al., 2017).

This promotes an anti-inflammatory state and implicates CD73 in the healing functions of mesenchymal stem cells.

Interestingly, a functionally significant upregulation of CD73 on epicardium-derived cells (EPDCs) following MI promotes the release of pro-inflammatory cytokines and pro-fibrogenic matrix proteins (Hesse et al., 2017). EPDCs, which are normally quiescent in the adult heart, are activated and give rise to multiple cell types following ischemic heart injury. The increased production of CD73-generated adenosine from EPDCs leads to $A_{2B}R$ activation, which stimulates the release of pro-inflammatory cytokines (IL-6, IL-11, and VEGF) (Hesse et al., 2017). Although the overall *in vivo* evidence thus far suggests a protective, anti-inflammatory function of CD73, a critical understanding of the cell-specific mechanisms for how CD73 regulates cardiac remodeling or healing after MI may promote development of potential therapeutic avenues around CD73 function.

CHAPTER 3: CD73 FUNCTIONS IN THE CENTRAL NERVOUS SYSTEM

CD73 expression and distribution in the central nervous system (CNS).

Immunohistochemical localization of CD73 in mouse brain performed in two independent studies revealed intense staining in the striatum (Augusto et al., 2013; Kulesskaya et al., 2013) as well as global pallidus, choroid plexus and meninges (Kulesskaya et al., 2013). Of note, these structures did not show any positive signal with an anti-CD73 antibody in brain sections from *Nt5e*$^{-/-}$ mice, establishing specificity of the assay (Kulesskaya et al., 2013). Biochemically CD73 appears to contribute ~90% of the 5'-nucleotidase activity across the brain, but the spatial distribution and the identity of the predominant cell types that exhibit CD73 activity in the mouse brain was not determined (Kulesskaya et al., 2013). The Human Protein Atlas reports highest expression of CD73 in the cerebellum (Purkinje and granular cell layer) and cortical endothelial cells (Uhlen et al., 2015), which is in stark contrast to previous work in mouse brain reporting complete lack of 5'-nucleotidase activity in the granular layer of the cerebellum and blood vessels (Langer et al., 2008). Several potential factors may account for this discrepancy, including assay sensitivity (e.g. antibody detection versus enzymatic activity) and differences in CD73 distribution between mouse and human brain. The cell type specific distribution of CD73 in the mouse spinal cord was characterized with the use of specific neuronal subtype markers, showing strong expression on L3-L5 dorsal root ganglion neuron membranes, particularly on the subset of neurons that express nociceptive neuron markers (IB4, Mrgprd, CGRP, and TRPV1) (Sowa, Taylor-Blake, & Zylka, 2010). Axon terminals in lamina II of the spinal cord express

CD73, along with another ecto-nucleotidase, prostatic acid phosphatase (PAP). Similar to CD73, PAP hydrolyzes AMP to produce adenosine and both proteins and their corresponding activities decrease in response to nerve injury (Sowa, Taylor-Blake, et al., 2010). Since the activities of CD73 and PAP enzymes are sensitive to the pH, Sowa et al. proposed that functional predominance of one enzyme may be relevant under certain conditions, such as during inflammation or tissue acidosis (Sowa, Taylor-Blake, et al., 2010). Multiple studies to date have implicated CD73 in major CNS functions, including locomotion and behavior (Augusto et al., 2013; Kulesskaya et al., 2013), memory functions and plasticity (Blundon et al., 2017; Zlomuzica, Burghoff, Schrader, & Dere, 2013), sleep regulation (Zielinski, Taishi, Clinton, & Krueger, 2012), thermoregulation (Muzzi et al., 2013), host-pathogen interactions during brain infection (Mahamed, Mills, Egan, Denkers, & Bynoe, 2012), inflammation (Mills et al., 2008; Petrovic-Djergovic et al., 2012; Xu et al., 2018), and nociception. We highlight several studies where the mechanisms of CD73 have been well described.

CD73 controls locomotion. In the striatum CD73 is closely associated with the $A_{2A}R$ in the post-synaptic compartment, and this interaction appears to be important for controlling locomotion because *Nt5e*$^{-/-}$ mice have decreased locomotor activity compared to WT mice after repeated amphetamine administration, phenocopying $A_{2A}R$ knockout mice (Augusto et al., 2013). In contrast, baseline locomotion in the *Nt5e*$^{-/-}$ mice is elevated when measured in the elevated plus maze, open field test, circadian activity test, and monitoring in the housing cages, although the mechanisms behind these observations were not investigated (Kulesskaya et al., 2013). Therefore, the effect of CD73 on locomotion appears to be context specific, and likely mediated by the spatiotemporal dynamics of the signaling pathways over which CD73 exerts control, together with other ecto-nucleotidases and adenosine receptor subtypes.

Thalamic CD73 tunes the plasticity of the auditory cortex. The auditory cortex of adults, unlike newborns, lacks the plasticity required to tune neural circuitry upon passive exposure to auditory inputs from the environment (Kehayas & Holtmaat, 2017). Recently, Blundon et al. (Blundon et al., 2017) identified CD73-generated adenosine and subsequent A_1R activation to be a key mechanism for age-dependent decline in auditory cortex plasticity. Genetic deletion of the A_1Rs from the auditory thalamus of mature mice promoted plasticity of the auditory cortex after passive tone exposure (Blundon et al., 2017). Compared to neonates, thalamic expression of CD73 in mature mice was significantly elevated, which paralleled increased adenosine production (Blundon et al., 2017). In mature *Nt5e*$^{-/-}$ mice exposed to a pure tone there was induction in auditory cortex plasticity, and tone-exposed *Nt5e*$^{-/-}$ mice distinguished frequencies better than tone-naive *Nt5e*$^{-/-}$ mice (Blundon et al., 2017). These results may have implications for restoring cortical plasticity via CD73 manipulations in learning and other contexts, such as recovery after stroke.

CD73 on tissue-resident cells regulates CNS inflammation. Given the central role of adenosine as an immunomodulator, several studies have addressed the function of CD73 in brain inflammation (Mills et al., 2008; Petrovic-Djergovic et al., 2012; Xu et al., 2018). Using genetic and pharmacological approaches, Petrovic-Djergovic and colleagues demonstrated a protective role of CD73 in neuroinflammation due to ischemic stroke (Petrovic-Djergovic et al., 2012). Specifically, *Nt5e*$^{-/-}$ mice were more susceptible to ischemic stroke injury, and the ischemic tissue had increased influx and activation of macrophages and pro-inflammatory markers, such as IL1-β, IL6, and TNF-α (Petrovic-Djergovic et al., 2012). This effect was reversed by administration of soluble CD73, suggesting that the effect was adenosine-mediated (Petrovic-Djergovic et al., 2012). Furthermore, bone marrow transplantation experiments demonstrated

that the protective effect of CD73 stemmed from tissue-resident cells, as opposed to CD73 on circulating immune cells that infiltrated after the injury (Petrovic-Djergovic et al., 2012). While the specific role of CD73 on astrocytes has not been examined, it is possible that astrocytes play an key role in this model, since astrocytes contribute to CD73-generated adenosine (Chu, Xiong, & Parkinson, 2014) and control neuronal injury following ischemic stroke (Takano, Oberheim, Cotrina, & Nedergaard, 2009).

Surprisingly, CD73 is pro-inflammatory in a mouse model of experimental autoimmune encephalomyelitis (EAE), which mimics inflammation associated with multiple sclerosis (Mills et al., 2008). Whereas WT mice displayed weak tail and partial hind limb paralysis and weak tail by 3 weeks of disease onset, the *Nt5e*$^{-/-}$ mice only had a weak tail and the disease did not worsen over time (Mills et al., 2008). Lymphocyte infiltration into the brain was significantly blunted in the *Nt5e*$^{-/-}$ mice, implicating CD73 as a facilitator for the entry of pathogenic T cells into the CNS (Mills et al., 2008). Similar to the stroke model, adoptive transfer studies demonstrated a role for CD73 on non-hematopoietic cells, potentially choroid plexus epithelial cells, which expressed CD73 in the WT mice (Mills et al., 2008). Modulation of blood-brain barrier function via CD73-generated adenosine and activation of A_1R and $A_{2A}R$ receptors in one potential mechanism behind the increased lymphocyte infiltration and inflammation in the EAE model (Carman, Mills, Krenz, Kim, & Bynoe, 2011). Combined, these two studies demonstrate that CD73 can exert pro- or anti-inflammatory effects in the brain, depending on the specific inflammatory condition and the cell types involved, which is an important consideration for potential therapeutic applications of CD73 modulators in CNS inflammation.

Neuronal CD73 regulates nociception. CD73 and two additional nucleotidases (PAP and tissue non-specific alkaline phosphatase) generate extracellular adenosine in the spinal cord to

regulate the function of pain sensing neurons (Street et al., 2013; Street & Zylka, 2011). A series of elegant studies on *Nt5e*$^{-/-}$ mice, utilizing supplementation with soluble mouse CD73 enzyme, demonstrated that CD73 plays a key role in regulating nociception in the mouse spinal cord (Sowa, Voss, & Zylka, 2010; Xu et al., 2018). Intrathecal administration of soluble mouse CD73 protein elicited dose-dependent and long-lasting (2 days) antinociceptive effects in response to heat-induced pain (Sowa, Voss, et al., 2010). Similarly, soluble CD73 had antinociceptive effects in inflammatory and neuropathic pain models (Sowa, Voss, et al., 2010). At the molecular level, these effects were the result of adenosine-dependent A_1R activation, since soluble CD73 did not produce antinociceptive effects in A_1R-/- mice (Sowa, Voss, et al., 2010). The relative contribution of CD73 on hematopoietic cells versus neurons in the inflammatory pain models is not presently clear, because studies using bone marrow chimera have not been performed. However, upon spinal cord injury, CD73 promotes polarization of macrophages and microglia to the M2 anti-inflammatory phenotype, suggesting that immune cells may play at least a partial role in the protective mechanism of CD73 in pain models (Xu et al., 2018).

CHAPTER 4: CD73 FUNCTIONS IN THE LUNG

CD73 expression and distribution in the lung. Together with tissue non-specific alkaline phosphatase, CD73 is known to be the major regulator of adenosine production on airway surfaces (Picher, Burch, Hirsh, Spychala, & Boucher, 2003). Human Protein Atlas reports high CD73 expression on human pneumocytes, which exhibit both cytoplasmic and membrane distribution. CD73 is also expressed and enzymatically active on primary human nasal and bronchial epithelial cells where it controls proper cilia beating frequency and ion transport, thus demonstrating its role in mucociliary clearance which is protective in the development of infectious lung diseases (Picher et al., 2003).

CD73 promotes maintenance of tissue barrier function in the lungs during hypoxia and hyperoxia. One of the earliest phenotypes reported using the global CD73-null mice showed vascular leakage in response to normobaric hypoxia in multiple tissues, which was most pronounced in the lung (Thompson et al., 2004). Hypoxia-induced vascular leakage was only partially reversed by adenosine receptor agonists and administration of soluble CD73, leaving open the possibility for additional non-enzymatic functions of CD73 that may be compromised in the *Nt5e*$^{-/-}$ mice. In humans, acute respiratory distress syndrome (ARDS) leads to pulmonary vascular leakage and clinical trials utilizing recombinant IFN-β1 (FP-1201) as a treatment strategy have revealed the potential to induce CD73 expression in the lungs and reduce mortality in patients with ARDS (Bellingan et al., 2017; Bellingan et al., 2014). Specifically, these studies

demonstrated that CD73 was significantly upregulated *ex vivo* in peri-bronchiolar vessels of cultured human lung tissue in response to IFN-β1 treatment, and that IFN-β1 attenuated 28-day mortality (8% of IFN-β1 treated patients died, versus 32% in the control group) (Bellingan et al., 2014). CD73 is highly upregulated in response to hyperoxia, and *Nt5e*$^{-/-}$ mice develop more severe pulmonary edema during hyperoxic lung injury. The latter effect, which is also phenocopied in $A_{2A}R^{-/-}$ mice, is attributed to loss of barrier function in the setting of decreased adenosine production (Davies et al., 2014).

CD73 protects against lung infection, inflammation and fibrosis. CD73-null mice exhibit increased weight loss and inflammation in response to intratracheal administration of lipopolysaccharide (LPS) (Ehrentraut et al., 2013) concomitant with significant transcriptional upregulation of TNFα, IL-1β, and IL-6. These effects were attributed to adenosine generation by regulatory T cells and the phenotypes of the *Nt5e*$^{-/-}$ mice were partially rescued by administration of soluble CD73. Deletion of CD73 does not affect baseline adenosine levels in the lungs, but it prevents adenosine production in response to bleomycin treatment (Volmer, Thompson, & Blackburn, 2006). Consequently, bleomycin treated *Nt5e*$^{-/-}$ mice exhibit enhanced inflammation, collagen production, and more severe fibrosis, which were attenuated by intranasal administration of exogenous nucleotidase. Treatment with the CD73 inhibitor APCP or the non-selective adenosine receptor antagonist CGS 15943 caused increased susceptibility of mice to *S. pneumoniae* lung challenge, although the effect was more pronounced upon CD73 inhibition (Bou Ghanem et al., 2015).

CD73 may contribute to asthma pathogenesis. Although CD73 function is known to be critical for regulating airway diseases, its involvement in asthma is poorly understood. CD73 expression is upregulated in the lungs of ovalbumin-treated mice (Bentley et al., 2010), and a

single study reported that *Nt5e*$^{-/-}$ mice are protected against ovalbumin-induced airway hyperresponsiveness and inflammation (Schreiber, Castrop, & Kunzelmann, 2008). To our knowledge, CD73 expression and activity in asthma patients has not been examined, with the exception of demonstration that BAL fluid from a patient with asthma contained a subpopulation of CD73-expressing cells that were thought to represent a mesenchymal progenitor cell type able to differentiate into fat, bone or cartilage (Bentley et al., 2010). Whether CD73 is simply a marker of these cells, or an active player in MSC-mediated regulation of inflammatory and fibrotic responses in the lungs remains to be determined.

CHAPTER 5: CD73 FUNCTIONS IN THE KIDNEY

CD73 expression and distribution in the kidney. The enzymatic activity of CD73 in the kidneys is nearly highest of all the tissues in the body (Colgan et al., 2006; Thompson et al., 2004). In rat kidneys CD73 decorates the apical membranes of the proximal convoluted tubule, intercalated cells of the distal nephron, and is also expressed in the peritubular space (Shirley, Vekaria, & Sevigny, 2009). Similar to other tissues, renovascular function is under the control of adenosine produced by both CD73 and TNAP (Jackson, Cheng, Verrier, Janesko-Feldman, & Kochanek, 2014).

CD73 regulates renal ischemia-reperfusion (I/R) injury. Adenosine receptor signaling promotes renal I/R injury, based on findings that $A_{2B}R^{-/-}$ are protected in I/R (Grenz et al., 2008). Additionally, genetic deletion or pharmacological inhibition of CD73, CD39, or both in mice revealed that CD73 and CD39 exacerbate I/R injury (Rajakumar et al., 2010; Roberts et al., 2013). Proximal tubular CD73 in particular is important for regulating renal I/R injury (Sung et al., 2017). In contrast to this reported injury-promoting role, CD73 plays a protective role in the context of isoflurane-mediated protection against renal I/R injury. Mice pretreated with a CD73 inhibitor or an adenosine receptor antagonist are insenstitive to this isofluorane-mediated protection (Kim et al., 2013).

CD73 regulates hypertension-associated renal injury. CD73 is upregulated and generates adenosine in a model of hypertensive nephropathy induced by angiotensin II (Zhang et al., 2013). CD73-generated adenosine promotes hypertension associated with chronic kidney disease through $A_{2B}R$, which is driven by hypoxia-inducible factor-α (Zhang et al., 2013). In

contrast to this hypertension promoting role, CD73 is protective through $A_{2B}R$ signaling in the context of diabetic neuropathy in the mouse kidney (Tak et al., 2014). CD73 is also protective in the rat kidney in a diabetic neuropathy model induced via streptozotocin (Taskiran et al., 2016). Furthermore, pharmacological antagonists for A_3R alleviate fibrosis driven by diabetic neuropathy in rat kidneys (Kretschmar et al., 2016).

Other functions of CD73 in the aging and injured kidney. CD73 knockout mice exhibit spontaneous proteinuria and renal functional deterioration as they age because of an autoimmune inflammation affecting the glomerular endothelium (Blume et al., 2012). Pharmacological inhibition or genetic deletion of CD73 in mice leads to more severe kidney injury in sepsis models (via cecal ligation) compared to WT mice (Hasko et al., 2011). Polymorphisms in *NT5E* have been implicated in the development of calcific uremic arteriolopathy in dialysis patients, which is associated with end stage kidney disease (Rothe et al., 2017).

CHAPTER 6: CD73 FUNCTIONS IN THE LIVER

CD73 expression and distribution in the liver. In the normal human liver CD73 is expressed on the apical membrane of hepatocytes as well as endothelial cells, which express CD73 at lower levels compared to hepatocytes (Matsuura, Eto, Kato, & Tashiro, 1984). During myofibroblast differentiation of activated hepatic stellate cells in culture there is transcriptional upregulation of *NT5E* (Fausther, Sheung, Saiman, Bansal, & Dranoff, 2012), but this is in contrast to significant downregulation observed in human liver fibrosis, regardless of etiology or severity (Snider et al., 2013). CD73 recently has emerged as a critical regulator of hepatocyte responses to different forms of injury (Hart et al., 2008; Peng et al., 2009; Peng et al., 2008; Snider et al., 2013), illuminating common disease mechanisms that may be exploited therapeutically.

CD73 regulates hepatic fibrosis. *Nt5e*$^{-/-}$ mice were previously reported to be resistant to hepatic fibrosis (Peng et al., 2008) induced by carbon tetrachloride (CCl4) or thioacetamide (TAA), which is surprising because genetic or pharmacologic inhibition of adenosine $A_{2A}R$ causes exacerbated liver injury in inflammation and fibrosis injury models (Chan et al., 2006; A. Ohta & Sitkovsky, 2001). If adenosine has a protective role in hepatic fibrosis, then removal of the major enzymatic source of extracellular adenosine (CD73) should exacerbate the pathology, but the opposite is seen. One of several possible interpretations is that constitutive *Nt5e* deletion eliminated an adenosine-independent function of CD73 that promotes hepatic fibrosis. If that is the case, it may also help explain the resistance of CD73$^{-/-}$ mice to ethanol-induced steatosis (Peng et al., 2009) and Mallory-Denk body-associated hepatocellular injury (Snider et al., 2013)

CD73 regulates hepatic steatosis. An early phenotype of ethanol consumption that damages the liver is the development of hepatic steatosis, which is the accumulation of fat in the liver. WT mice develop hepatic steatosis while mice lacking CD73 or adenosine A_1 or A_{2B} receptors are protected after alcohol treatment (Peng et al., 2009). Additionally, *in vitro* studies using cultured murine hepatocyte cell lines supported these findings, suggesting adenosine generated by ethanol metabolism plays an important role in ethanol-induced hepatic steatosis (Peng et al., 2009). These data demonstrate that CD73 is important in the role acute liver diseases, and it would be beneficial to determine if CD73 can mediate the progression into chronic liver diseases.

CD73 regulates hepatic fibrosis. Several studies have shown the importance of CD73 in chronic liver disease models, such as liver fibrosis. For example, studies utilizing chemical injury (carbon tetrachloride or thioacetamide treatment) for the induction of liver fibrosis in mice illustrated that *Nt5e*$^{-/-}$ mice were protected from the development of liver fibrosis or cirrhosis, while WT mice developed fibrosis and cirrhosis(Peng et al., 2008). Liver fibrosis is accompanied by the activation of liver myofibroblasts, that typically originate from hepatic stellate cells and portal fibroblasts (Iwaisako et al., 2014). Although CD73 is expressed in murine liver fibrosis models and liver myofibroblasts, *NT5E* is transcriptionally down-regulated via mediation by Elf2-like transcription factors in mouse-activated liver myofibroblasts (Fausther, Lavoie, Goree, & Dranoff, 2017). Conversely, other studies have shown *NT5E* transcriptional up-regulation through promoter response elements for SP1 and SMAD transcription factors (Fausther et al., 2012).

CD73 regulates hepatocyte injury. In the context of hepatic ischemia, CD73 is induced at the transcriptional and protein level (Hart et al., 2008) and serves a protective function. *Nt5e*$^{-/-}$

mice develop more severe injury, which can be reversed by administration of soluble enzyme (Hart et al., 2008). Based on this a proposed method to elicit hepatoprotection during hepatic ischemia/reperfusion would be to inhibit adenosine uptake (Zimmerman et al., 2013). Equilibrative nucleoside transporters (ENTs) are responsible for the uptake of adenosine, and transcriptional downregulation of ENTs are correlated with ischemia and reperfusion during human liver transplantation and during murine liver ischemia and reperfusion (Zimmerman et al., 2013). Ultimately, these studies provide the framework for potential therapies to manage ischemia and reperfusion by maintaining extracellular adenosine levels.

The formation of hepatocyte Mallory-Denk bodies (MDBs), which are aggregates of keratins 8 and 18 (K8/K18), ubiquitin, and the ubiquitin-binding protein p62, are generally associated with the development of chronic liver diseases (Snider et al., 2013). We previously have demonstrated that CD73 modulates the formation of MDBs in a strain-related manner (Snider et al., 2013). In mice livers that lacked CD73, chemical-induced liver injury (in the form of MDBs) was prevented, but this protection was not observed in WT mice under the same experimental conditions (Snider et al., 2013).

CHAPTER 7: CD73 FUNCTIONS IN THE IMMUNE SYSTEM

CD73 expression on immune cells. The role of CD73 has been most extensively studied in context of the immune system (Antonioli, Pacher, et al., 2013; Resta, Yamashita, & Thompson, 1998). CD73 is expressed in macrophages, monocytes, dendritic cells, and neutrophils (Antonioli, Pacher, et al., 2013; Resta & Thompson, 1997; Resta et al., 1998; Saze et al., 2013). It is highly expressed on lymphocytes, particularly T cells and B cells (Conter, Song, Shlomchik, & Tomayko, 2014; Kaku, Cheng, Al-Abed, & Rothstein, 2014; Saze et al., 2013). In T cells, thymic γδ lineage commitment relies on T cell receptor ligand-induction of CD73 expression.

CD73 regulates immune cell migration. The expression of CD73 on the cell surface has been shown to play a key role in immune cell migration in response to inflammatory stimuli. Upon LPS treatment in mice, CD73 on endothelial cells restricts the migration of lymphocytes into the draining lymph nodes through the activation of A_2Rs (Takedachi et al., 2008; Thompson et al., 2008). Mills et al showed that the infiltration of T cells into the central nervous system during experimental autoimmune encephalomyelitis in mice is regulated by CD73 that is highly expressed in the choroid plexus (Mills et al., 2008). In the absence of CD73 in the immune cells, the number of infiltrating lymphocytes increases and further exacerbates the inflammation or condition. CD73 KO mice that were infected with *Toxoplasma gondii* showed a higher propensity for neutrophil and T cell infiltration, inducing an immune-mediated pathology (Mahamed, Toussaint, & Bynoe, 2015). The administration of the adenosine receptor agonist, NECA attenuated the severity of inflammation, suggesting a key role of the adenosine-

generating function of CD73. Similarly, $CD4^+$ T cells that lack CD73 produce a stronger immune response against bacterial infection, and generate pro-inflammatory cytokines like TNFα, IFN γ, IL17A, and keratinocyte chemoattractant.

CD73 regulates chronic inflammation. In the germinal center, follicular dendritic cells interact with B cells via CD73 and induce maturation (Airas & Jalkanen, 1996). Interestingly, B1 cells, which are a part of the humoral response, express a 5-fold increase in CD73 compared to the adaptive immune B2 cells (Conter et al., 2014). Allard et al further showed that the lack of CD73 and subsequent adenosine signaling in B1 cells delayed isotype switching from the primary response IgM antibody to the pro-inflammatory IgG3 antibody (Allard, Charlebois, Gilbert, Stagg, & Chrobak, 2018). However, this delay in isotype switching did not alter the protective response to infection. In a chronic state of inflammation, CD73-expressing T follicular helper cells regulate the differentiation and maintenance of B cells in to plasma cells, suggesting an important role of CD73 in establishing humoral immunity (Conter et al., 2014). While macrophages and monocytes express cell surface CD73 in both mice and humans, their polarization towards a pro-inflammatory M1 or anti-inflammatory M2 phenotype is independent of CD73 (Eichin, Laurila, Jalkanen, & Salmi, 2015).

Genetic deletion of CD73 promotes T cell expansion, and production of pro-inflammatory cytokines IFNγ and IL-6 in a mice model of graft vs host disease (GvHD) (Tsukamoto et al., 2012). Specifically, blocking the enzymatic activity of CD73 induced a stronger alloreactive T cell activity, suggesting that the adenosine-generating function is important in the progression of GvHD (Wang et al., 2013). Thus, these suggest an important function of CD73 in the immune cells in attenuating the severity of inflammation or condition through the adenosine signaling.

CHAPTER 8: CD73 FUNCTIONS IN OTHER TISSUES AND CELL TYPES

Gastrointestinal system. $CD4^+$ T cells that lack CD73 produce a stronger immune response against bacterial infection, and generate pro-inflammatory cytokines like TNFα, IFN-γ, IL17A, and keratinocyte chemoattractant. This type of cytokine milieu resulted in more severe *Helicobacter felis*-induced gastritis or murine salmonellosis compared to the WT mice due to the failure of T regulatory cells lacking CD73 to attenuate inflammation. However, there was a significant enhancement of bacterial clearance in CD73 KO mice that were infected with *H. felis* or *Salmonella* (Alam et al., 2014; Alam et al., 2009). CD73 KO mice also exhibited a more severe condition of experimental inflammatory colitis compared to WT mice (Kaku et al., 2014). Upon adoptive transfer of CD73-expressing B1 cells from WT mice to CD73 KO mice, the disease severity was reduced (Kaku et al., 2014).

Bone. The role of adenosine signaling in the development and differentiation of bone tissue, osteoblasts, and osteoclasts has been implicated in several studies. For example, a study demonstrated that adenosine receptor activation induced mitogenesis and triggered a protective response in regard to cell death (Fatokun, Stone, & Smith, 2006; Shimegi, 1998). Additionally, in human primary osteoblast cells it was shown that pharmacological activation of adenosine receptors promoted proliferation, and proliferation was halted with antagonism (Costa et al., 2011). CD73 is a known marker of osteochondroprogenitor cells, which readily form bone after transplantation (Singh et al., 2015) and CD73-null mice develop bone deficiencies, such as osteopenia (Takedachi et al., 2012). Furthermore, *in vitro* studies using MC3T3-E1 cells demonstrated CD73-generated adenosine signals through the adenosine A2B receptor to

modulate osteoblast differentiation. Recently, it was demonstrated that CD73-generated adenosine in human amniotic fluid stem cells enhances osteogenic potential via TGFβ induction (Hau et al., 2017). Overall, CD73 and CD73-generated adenosine are important in the process of bone development and growth. In terms of pathologic conditions, CD73-deficient mice are significantly more susceptible to developing collagen-induced arthritis compared to wild-type mice (Chrobak et al., 2015). In the absence of CD73, there is enhanced production of proinflammatory cytokines in the joints and augmented Th1 T cell responses, leading to joint destruction. Importantly, using bone marrow transplants it was shown that the protective activity of CD73 originated from nonhematopoietic cells (Chrobak et al., 2015).

Inflammation of the eye. CD73 marks an intermediate stage in γδ T cells from which their ability to express effector genes IFNγ or IL-17 is resolved (Coffey et al., 2014). Related to this function, it was reported that CD73 expression on γδ T cells is dynamically regulated during experimental autoimmune uveitis (EAU) and that low CD73 expression on γδ T cells correlated with enhanced Th17 response-promoting activity. Mice lacking CD73 showed decreased susceptibility to developing EAU (Liang et al., 2016).

Reproductive tissues. In CD73-deficient mice, it was demonstrated that CD73-generated adenosine is important to initiate and maintain penile erections (Wen et al., 2011). In humans, the expression of ecto-nucleotidases, such as CD73, fluctuates in concert with the menstrual cycle and changes after menopause in the endometrium (Aliagas et al., 2013).

Skin and Muscle. *Nt5e*$^{-/-}$ mice have lower susceptibility to developing bleomycin-induced skin fibrosis (Fernandez et al., 2013). This response, also seen in the CD39-deficient mice, was associated with reduced expression of the profibrotic mediators, TGF-β1 and connective tissue growth factor, and decreased myofibroblast cell populations (Fernandez et al.,

2013). In *Nt5e*$^{-/-}$ mice that were fed a high fat diet, CD73 plays a protective role in the development of dyslipidemia and intramyocellular lipid accumulation in the muscle of mice (Burghoff et al., 2013). Ultimately, CD73 function is critical in various tissues and processes in the body, therefore, it is essential to potentially exploit CD73 and adenosine signaling in the development of novel therapies that can be utilized to improve health.

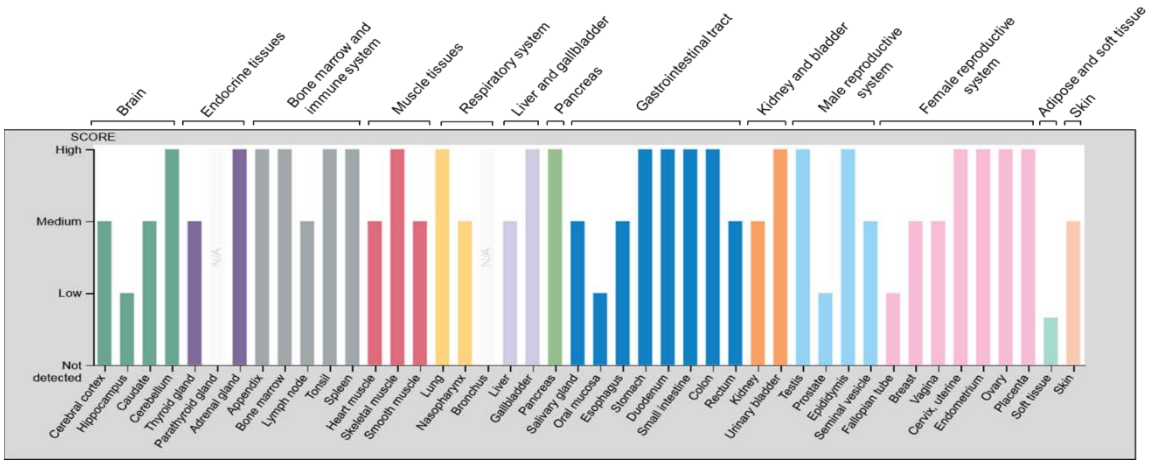

Figure 1. Relative levels of CD73 protein expression across human organ systems and tissues. The expression data on CD73 (Gene: *NT5E*) were collected from the Human Protein Atlas Project (https://www.proteinatlas.org/). Note that CD73 is ubiquitous and abundantly expressed in most tissue types.

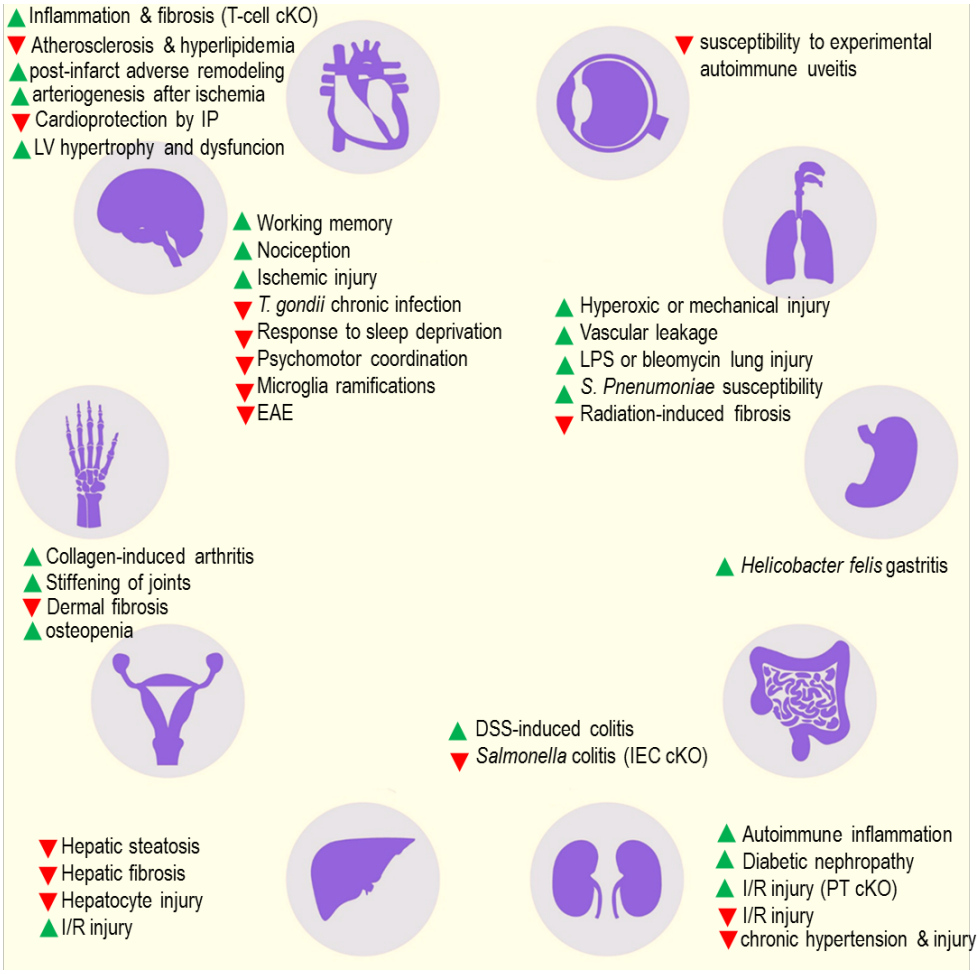

Figure 2. Reported phenotypes of the CD73 knockout mice. Unless otherwise specified, all studies utilized the whole body knockout model. Green upward pointing arrow denotes that the response was elevated in the CD73 knockout mouse; red downward arrow denotes that the response was decreased in the CD73 knockout mouse. In cases where conditional knockouts (cKO) were used, this is specified in the parenthesis.

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