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Review Article

Ectonucleotidases in Solid Organ and Allogeneic Hematopoietic Cell Transplantation

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Extracellular nucleotides are ubiquitous signalling molecules which modulate distinct physiological and pathological processes. Nucleotide concentrations in the extracellular space are strictly regulated by cell surface enzymes, called ectonucleotidases, which hydrolyze nucleotides to the respective nucleosides. Recent studies suggest that ectonucleotidases play a significant role in inflammation by adjusting the balance between ATP, a widely distributed proinflammatory danger signal, and the anti-inflammatory mediator adenosine. There is increasing evidence for a central role of adenosine in alloantigen-mediated diseases such as solid organ graft rejection and acute graft-versus-host disease (GvHD). Solid organ and hematopoietic cell transplantation are established treatment modalities for a broad spectrum of benign and malignant diseases. Immunological complications based on the recognition of nonself-antigens between donor and recipient like transplant rejection and GvHD are still major challenges which limit the long-term success of transplantation. Studies in the past two decades indicate that purinergic signalling influences the severity of alloimmune responses. This paper focuses on the impact of ectonucleotidases, in particular, NTPDase1/CD39 and ecto-5′-nucleotidase/CD73, on allograft rejection, acute GvHD, and graft-versus-leukemia effect, and on possible clinical implications for the modulation of purinergic signalling after transplantation.

1. Introduction

Purinergic signalling has been recognized in the past decades as one of the important mediator pathways regulating cellular functions under physiological and pathological conditions. There are three major components of purinergic signalling: nucleotides, purinergic receptors, and ectonucleotidases. Nucleotides such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP), or uridine diphosphate (UDP) are released by a variety of cell types especially under cell stress conditions. Purinergic receptors can be divided in two major groups: nucleotide (P2) receptors and nucleoside/adenosine (P1) receptors. On the one hand, P2 receptors include 7 ligand-gated ion channels (P2X receptors) and 8 G-protein-coupled receptors (P2Y receptors). On the other hand, four P1 receptors have been described so far: A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor (AR). Purinergic signalling is regulated by ectonucleotidases, enzymes located on the cell surface which hydrolyze extracellular nucleotides and eventually metabolize them to the respective nucleosides [1]. By regulating the levels of extracellular nucleotides and nucleosides, ectonucleotidases are involved in numerous physiological and pathological responses, such as inflammation [2], pain [3], thromboregulation [4], tumor growth, and metastasis [5, 6].

Researchers in this field have identified four major families of ectonucleotidases: NTPDase family (nucleoside triphosphate diphosphohydrolases), nucleotide pyrophosphatase/phosphodiesterase-(NPP)-type ecto-phosphodiesterases, alkaline phosphatases and ecto-5′-nucleotidase (CD73). Other enzymes capable of metabolizing and interconverting extracellular nucleotides include nucleoside diphosphate kinases, adenylate kinase, ecto-ADP-ribosyltransferases, adenosine deaminase, and purine nucleoside phosphorylase [7]. Emerging evidence shows that prostatic acid phosphatase (PAP) also has a membrane-bound form, which can hydrolyze adenosine monophosphate (5′-AMP) to adenosine [8].

Solid organ and allogeneic hematopoietic cell transplantation (allo-HCT) are increasingly performed treatment modalities for a large variety of diseases. Despite improved immunosuppressive medication, allograft rejection after solid organ transplantation and GvHD after allo-HCT are still major complications which prevent a broader application of these therapeutic options. Allograft rejection and GvHD are both based on recognition of alloantigens between donor and recipient leading to tissue destruction by activated cells from the adaptive immune system. These responses are regulated by diverse cell types, cytokines, chemokines, and soluble mediators. There is increasing evidence that purinergic signalling is involved in inflammatory reactions after transplantation, so that ectonucleotidases modulate the severity of alloimmune responses and also of ischemia-reperfusion injury by regulating the levels of extracellular nucleotides and nucleosides.

This review concentrates on the role of ectonucleotidases, especially NTPDase1 (CD39) and ecto-5′-nucleotidase (CD73), in solid organ transplantation and allo-HCT and their function in clinically important reactions such as delayed graft function (DGF), allograft rejection, acute GvHD, and graft-versus-leukemia (GvL) activity.

2. Ectonucleotidase Families

The first ectonucleotidase family, the NTPDase family, includes enzymes with common motifs in their protein sequences which are able to hydrolyze extracellular ATP and other NTPs as well as NDPs [9]. NTPDases are expressed not only in mammals but also in plants, worms, and protozoa. So far, eight human NTPDases have been identified (NTPDase1–8). Four of these enzymes are membrane-bound with their active sites on the cell surface: NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), and NTPDase8 (ecto-ATPDase). They have different tissue distribution [7] and can also be simultaneously expressed by the same cell type, indicating that purinergic signalling is a subject of complex regulation. NTPDase1 is expressed by murine and human regulatory T cells (Tregs) [10], neutrophils [11], lymphocytes [12], endothelial and epithelial cells [9, 13], mesenchymal stromal/stem cells (MSCs) [14], smooth muscle cells [15], and other cell populations. NTPDase2 is present in murine solid organs such as pancreas and salivary gland [16] as well as in neoplasms like mouse hepatoma [17] and human small cell lung carcinoma [18]. Additionally, it has been detected on the blood vessel adventitia [19] and on glial cells [20, 21]. NTPDase3 expression has been observed on human bronchial epithelial cells [22], dorsal root ganglion cells [23], neurons in the rat brain [24], Langerhans islet cells, and cells from the gastrointestinal mucosa in mice [25]. Finally, expression studies show that NTPDase8 is present in human and rat liver tissue and bile canaliculi [26, 27], as well as in the porcine kidney tubules [27]. These four ectonucleotidases have similar molecular sizes (500 kDa) with variable amount of glycosylation and their catalytic capacity and substrate affinity are different. NTPDase1, -3 and -8 can hydrolyze NTPs and NDPs whereas NTPDase2 metabolizes only NTPs [28].

NTPDases4–7 are integral membrane proteins as well but since they metabolize mostly only intracellular substrates, they do not belong to the ectonucleotidases.

The second family of ectonucleotidases, the nucleotide pyrophosphatase/phosphodiesterase (NPP)-type ecto-phosphodiesterases family comprises seven members-NPP1–7. These enzymes hydrolyze pyrophosphate or phosphodiester bonds in different types of molecules and regulate purinergic signalling, extracellular pyrophosphate levels, as well as nucleotide recycling and cell motility [29]. NPP1 and NPP3 convert NTPs directly to the respective nucleoside monophosphates, for example, ATP to 5′-AMP and are thus involved, similar to the NTPDases, in purinergic signalling. NPP2 metabolizes lysophosphatidylcholine and NPP6 and NPP7 have affinity towards choline phosphate esters. The substrate specificity of NPP4 and NPP5 remains unknown [30]. NPP1 and NPP3–7 are membrane bound and can be secreted to a variable extent, whereas NPP2 exists only in a secreted form [31]. NPP-type phosphodiesterases have broad tissue distribution. NPP1 has been found on human and murine immune cells, human bone and cartilage cells, in the distal convoluted tubules of the kidney, as well as on epithelial and endothelial cells [29]. Interestingly, NPP1 is not present in normal brain tissue, but it is abundantly expressed in human astrocytic brain tumors [32]. NPP2 is expressed in the brain, placenta, ovary, and small intestine [30], on epithelial cells, cartilage, and bone tissue [29], and accumulates in body fluids such as plasma and cerebrospinal fluid [30]. NPP3 has been implicated to play a role in allergic reactions, as it serves as a marker for basophils and mast cells [33]. So far, only little is known about the physiological functions of NPP4–7.

Thirdly, purinergic signalling is modulated by ecto-5′-nucleotidase/CD73. CD73 is a glycosyl phosphatidylinositol-anchored cell membrane enzyme which catalyzes the hydrolysis of extracellular nucleoside 5′-monophosphates to the respective nucleosides, in particular, of 5′-AMP to adenosine [34]. The mature CD73 protein consists of 548 amino acids and has a predicted molecular weight of 63 kDa [35]. It is ubiquitously expressed, including epithelial and endothelial cells, and also lymphocytes [36] and MSCs [37]. CD73 releases extracellular adenosine, a potent anti-inflammatory mediator which activates P1-type purinergic receptors (A_1, A_{2A}, A_{2B}, and A_3-AR). CD73 is often coexpressed with NTPDases or NPP-type ecto-phosphodiesterases and catalyzes the last step of the degradation of extracellular ATP. Its involvement in the regulation of physiological and pathological immune processes is discussed later.

Finally, alkaline phosphatases (ALPs) are enzymes which dephosphorylate numerous molecules, such as proteins, alkaloids, and nucleotides. They modulate purinergic signalling mainly by converting 5′-AMP into adenosine and can also hydrolyze NTPs. There are four ectoenzymes in the ALP family [38]: intestinal ALP, tissue nonspecific ALP detected in organs such as liver, bone, and kidney, placental ALP, and germ-cell ALP, expressed in testes and in malignant tumors.

Additionally, ALPs can dephosphorylate endotoxins [39] and serve as a host defence mechanism against pathogens. By converting the proinflammatory mediator ATP into the anti-inflammatory adenosine and by neutralizing lipopolysaccharide as an endotoxin, ALP has beneficial effects in an animal model of septic shock as its administration leads to improved gas exchange, reduced IL-6 serum levels and prolonged survival time [40].

Expression studies from different models show simultaneous expression of multiple ectonucleotidases on the same cell type. As stated above, CD73 is often coexpressed with NTPDases. This enzymatic cascade leads to metabolization of ATP, an important danger-associated molecular pattern (DAMP) inducing activation of the immune system and to release of extracellular adenosine which exerts immunosuppressive effects on distinct cell populations. A recent study on adenosine formation in the healthy rat liver shows that CD73 is partially coexpressed with NTPDase1, -2, and -3 [41]. However, these enzyme combinations appear to have different kinetics regarding ATP hydrolysis and adenosine release. The combination of NTPDase1/CD39 and CD73 results in immediate generation of adenosine, whereas this is not the case when CD73 is coexpressed with NTPDase2 or -3. These data suggest that the synergistic activity of CD39 and CD73 is a potent mechanism to convert the proinflammatory ATP into the anti-inflammatory adenosine and imply the particular combination of these two enzymes as a promising target for the modulation of immune responses, including alloimmunity.

3. Pathophysiology of Delayed Graft Function, Graft Rejection, Acute Graft-Versus-Host Disease and Graft-Versus-Leukemia Effect

Solid organ transplantation and allo-HCT are potentially curative therapeutic options for a broad spectrum of hereditary, non-malignant and malignant diseases. The first bone marrow transplantation took place in 1939, whereas the first successful solid organ transplantations were performed in the 1950s. Initial transplantation attempts remained ineffective due to the immune incompatibility between donor and recipient and the lack of adequate immunosuppressive drugs. Today, more than 60 years later, immunologic reactions between donor and host still remain one of the major causes of morbidity and mortality after solid organ transplantation and allo-HCT. Here we would like to summarize the major mechanisms leading to DGF, graft rejection, acute GvHD and GvL activity.

3.1. Delayed Graft Function. One of the major obstacles especially in the context of kidney transplantation is DGF. There are variable definitions of DGF including clinical criteria like the use of dialysis within the first week after transplantation but also pathological criteria such as signs of acute kidney injury [53]. Critical mechanisms leading to DGF are ischemia-reperfusion injury caused by decreased perfusion of the donor organs, release of inflammatory mediators due to brain or cardiac death, and cold or warm ischemia followed by reperfusion after transplantation. Reperfusion leads to infiltration of innate and adaptive immune cells which are attracted by chemotactic signals released from endothelial cells and by danger signals released from necrotic or apoptotic cells in the graft. There is evidence that macrophages, dendritic cells (DCs), and alloreactive T cells contribute to ischemia-reperfusion injury before inducing an allogeneic response [53].

3.2. Graft Rejection. The exact pathophysiologic mechanisms of graft rejection after solid organ transplantation have been extensively studied in the process of development of effective immunosuppressive drugs. Distinction between self and nonself is mediated in the first place by antigens from the major histocompatibility complex (MHC) or human leukocyte antigens (HLA). These can be recognized by immune cells of the host and initiate a cellular and humoral immune response. Graft rejection can be classified in three groups: hyperacute graft rejection, acute graft rejection, and chronic graft rejection.

Hyperacute graft rejection (HAR), also called humoral rejection or acute antibody-mediated rejection (AMR), is a very rapid antibody-mediated graft destruction which occurs within the first 24 hours, most often minutes to hours after transplantation [54]. It results from preformed donor-specific antibodies and leads to edema of the transplanted organ, platelet aggregation, formation of fibrin thrombi, neutrophil infiltration, and eventually endothelial damage, interstitial edema, haemorrhage, and infarction [55]. HAR plays a role in xeno- and allotransplantation, being one of the major factors limiting xenograft survival. Here, HAR is often based on the presence of Galactose-α(1, 3)-Galactose (αGal) epitopes on the porcine cells which are recognized by the human immune system. In humans, anti-αGal antibodies exist physiologically and are continuously produced due to antigenic stimulation by bacteria in the gastrointestinal tract (GIT) [56]. Recently, genetically modified galactosyl transferase knock-out pig organs have been developed and offer a possible new source of donor organs for human transplantation [57, 58]. Initial trials for xenograft transplantation of these organs into baboons show increased graft survival and reduced HAR [59, 60].

However, HAR plays a role not only in xenograft but also in allograft rejection caused by preformed antibodies against antigens such as HLA or AB0 molecules [55]. These antibodies destroy initially endothelial cells, which causes activation of the complement system with C4d deposition [61], infiltration of polymorphonuclear (PMN) leukocytes and macrophages and fibrinoid necrosis, resulting in thrombosis of the small blood vessels and early graft dysfunction [62]. In the last years, HAR has been a rare complication due to screening procedures for host antibodies against donor HLA prior to transplantation [63] but there are still case reports describing HAR in kidney [64], lung [65], and liver [66] transplantation.

Acute and chronic graft rejection are based on activation of the adaptive immune system by the recognition of non-self antigens after the transplantation. Three pathways for

alloantigen recognition have been established: the direct, the indirect, and the semidirect pathway. First, in the direct pathway recipient $CD8^+$ and $CD4^+$ cells recognize directly non-self MHC class I and II molecules respectively, expressed on donor antigen-presenting cells (APCs) present in the allograft. Second, in the indirect pathway, alloantigens have to be processed by recipient APCs and are then presented via MHC I and II to recipient $CD8^+$ and $CD4^+$ T cells [67]. Third, in the semidirect pathway host DCs acquire intact MHC:peptide complexes from donor APCs and present them to the recipient's T cells [68]. According to the current model, direct alloantigen recognition is involved mostly in acute graft rejection, whereas indirect alloantigen recognition is associated with chronic graft rejection.

Acute graft rejection occurs within the first 4–6 months after solid organ transplantation. It is initiated by T cells activated mostly via the direct pathway, for example, T cells are activated via their T cell receptor which recognizes nonself MHC molecules on the donor APCs. $CD4^+$ T helper cells can be activated by MHC class II molecules, whereas cytotoxic $CD8^+$ T cells recognize MHC class I molecules. In order to be completely activated, T cells require a second costimulatory signal, which is provided, for example, by the binding of CD28 on T cells to B7 molecules (CD80 or CD86) on APCs. This activation apparently takes place at least in part in the secondary lymphoid organs such as spleen, lymph nodes, Peyer's patches, and tonsils, as cardiac allografts transplanted into recipients lacking secondary lymphoid organs were not rejected [69]. However, secondary lymphoid organs are not absolutely required for the induction of an allogeneic response. Nonhematopoietic cells like vascular endothelial cells can activate $CD8^+$ T cells *in vivo* and *in vitro* and lead to allograft rejection even if the alloantigen is not expressed by hematopoietic APCs [70]. Activation of $CD4^+$ T helper cells leads to production of proinflammatory cytokines which enhance the proliferation and differentiation of $CD8^+$ cytotoxic T cells. After cytotoxic T cells are activated, they can migrate into the allograft and cause acute rejection by three major mechanisms. First, $CD8^+$ T cells secrete perforin, a pore-forming enzyme, and granzymes, which activate caspases and induce DNA fragmentation. Second, cytotoxic T cells kill target cells via Fas/FasL interaction. Third, they secrete cytotoxic proinflammatory cytokines such as IFN-γ and TNF-α which can lead to apoptosis [71]. Altogether, these mechanisms lead to tissue damage in the transplanted organ and eventually graft dysfunction. Cells from the innate immune system are also involved in acute graft rejection. There is evolving evidence that activation of innate immune cells via various pattern recognition receptors (PRRs) such as toll-like receptors (TLR), creates a proinflammatory microenvironment which supports the activation of the adaptive immune system. DCs as professional APCs contribute critically to T-cell activation and are an important target for potential immunosuppressive treatment. Transplant experiments with alymphoid $RAG^{-/-}$ donor and recipient mice which lack adaptive immune cells show that these mice upregulate cytokines such as IL-1β and IL-6 or chemokine receptors like CCR1-5 similar to transplants with wildtype mice [72]. Natural killer (NK) cells also play a supportive role for T-cell activation by secreting IFN-γ and TNF-α and amplifying early graft inflammation [73]. This early damage of the graft tissue leads to release of DAMPs (aka danger signals) from the dying cells like biglycan, hyaluronan, heparin sulphate, and some heat shock proteins which in turn activate APCs [73]. In conclusion, acute allograft rejection is a process undergoing complex regulation and involving distinct cell populations, proinflammatory cytokines, chemokines, and other mediators.

Chronic graft rejection occurs months to years after transplantation and is a main cause for long-term allograft dysfunction, but its exact pathophysiology remains still unclear. As explained above, the vascular endothelium is damaged in the early phase after transplantation by ischemia-reperfusion injury, complement activation, or formation of reactive oxygen species [74]. This is followed by increased infiltration of macrophages and elevated concentrations of proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and MCP-1 in the extracellular space. Endothelial cells also up regulate the secretion of IFN-γ, IL-1β, and TNF-α and subsequently show enhanced expression of adhesion molecules like ICAM-1 and VCAM-1 [75]. Allograft vessels additionally show elevated expression of the growth factors TGFβ, FGF, and PDGF. These mediators lead to cell proliferation and migration of smooth muscle cells, an important event in the development of intimal hyperplasia and early atherosclerosis [74]. The tissue remodelling leads eventually to vascular hypertrophy, sclerosis, fibrosis, and loss of graft function. Chronic rejection is accompanied by increased infiltration of the allograft with various subsets of immune cells. Memory $CD8^+$ T cells as well as B cells and cells from the innate immunity are involved in this process. For instance, B cells produce alloantibodies and also present alloantigens via MHC II to the infiltrating T cells. In the last years, there has been growing interest in the role of B cells in allograft rejection, for a recent review on humoral immunity in transplantation see [76].

3.3. Acute Graft-Versus-Host Disease. Allo-HCT is currently performed more than 25 000 times annually worldwide as a treatment mostly for patients suffering from hematological malignancies which are refractory to conventional chemotherapy. One of the frequent complications is the development of GvHD, a progressive systemic immunological disease. In 1966, Billingham defined three requirements for GvHD [77]. First, the graft must contain immunologically competent cells; second, the host must appear foreign to the graft due to histocompatibility differences; third, the host must be immunocompromised and, therefore, incapable of graft rejection. Based on the time point of manifestation, GvHD can be defined as acute (until day 100 after transplantation) or chronic (after day 100 after transplantation). In this section, we would like to focus on the pathophysiology of acute GvHD.

MHC mismatch between donor and recipient leads to activation of the donor immune system and an allogeneic response against host tissues. The incidence of acute GvHD

is related to the degree of mismatch between these molecules. For this reason, a suitable donor for allo-HCT nowadays would have the same HLA proteins like the host. However, without prophylaxis acute GvHD occurs in almost 40% of patients receiving HLA-identical grafts, due to genetic differences in the so-called "minor" histocompatibility antigens [78].

Manifestations of this disease are observed most frequently in organs with epithelial structure, such as skin, GIT and liver. The skin is affected in 81% of the patients with acute GvHD [79], the GIT is involved in 54% of the cases [79] with the typical symptom of diarrhoea, and also nausea, vomiting, and crampy abdominal pain [78]. Liver GvHD is present in 50% of the acute GvHD patients [79] and frequently manifests as painless jaundice with increase in alkaline phosphatase and bilirubin. According to the current pathogenetic model of GvHD, the disease develops in three stages. The first phase in acute GvHD is triggered by the preconditioning of the recipient for the transplant via administration of myeloablative radio- and/or chemotherapy. This treatment leads to necrotic and apoptotic cell death, particularly in the GIT, with subsequent activation of the immune system, release of proinflammatory cytokines like TNF-α and IL-1β, increased permeability of the gastrointestinal mucosa with translocation of pathogen-associated molecular patterns (PAMPs), such as bacteria-derived lipopolysaccharide (LPS), in the circulation. Furthermore, tissue destruction after the preconditioning treatment leads to release of specific DAMPs like ATP, uric acid, soluble matrix components, and others [80]. These signals activate the innate immune system of the host, especially the APCs, via interaction with purinergic, toll-like, or NOD-like receptors. In the second phase of acute GvHD, transplanted donor T cells interact with host-derived APCs, such as DCs [81]. Recent studies suggest that allorecognition and GvHD development can also be initiated by nonhematopoietic APCs [82]. Local proinflammatory cytokines produced in phase I serve as further stimuli for activation, differentiation, and proliferation [83]. In the third phase, the differentiated effector cells, mostly T cells, and also NK cells, macrophages, and neutrophils migrate after initial expansion to the target tissues of GvHD-skin, GIT and liver. There these cells lead directly or indirectly to tissue destruction. $CD8^+$ T cells induce direct cytotoxicity via Fas/FasL-signalling as well as via perforin and granzymes. Another mechanism inducing cell death is the secretion of proinflammatory cytokines by $CD4^+$, $CD8^+$ T cells, NK cells and mononuclear phagocytes. Cytotoxicity results in release of further DAMPs which perpetuate the tissue damage [84].

Our group has recently shown that ATP is released from dying cells after the preconditioning treatment prior to allo-HCT and that it serves as a critical danger signal for the activation of the immune system [50]. ATP binds to the purinergic P2X7 receptor on APCs and leads to increased expression of T-cell costimulatory molecules, followed by stronger activation of alloreactive T cells and more severe GvHD phenotype. As expected, blocking purinergic signalling via a P2X7 receptor antagonist or administration of soluble apyrase which metabolizes extracellular ATP significantly prolonged the survival of recipient mice, indicating a critical role for purinergic signalling in acute GvHD.

3.4. Graft-Versus-Leukemia Effect. Allo-HCT has one major therapeutic advantage in the treatment of hematologic malignancies. Immunologically competent cells in the graft can destroy any residual tumor cells via the GvL effect, thus preventing a relapse of the underlying disease. The GvL effect develops simultaneously with acute GvHD based on the same pathophysiological processes of allorecognition which are directed against the malignant cells. Donor lymphocyte infusions (DLIs) are another approach used to enhance the GvL effect in the case of relapse. This means that in allo-HCT, allorecognition leads on the one hand to increased morbidity by inducing GvHD, but it is on the other hand critical for relapse prevention via the GvL effect. Recent studies in the field of allo-HCT concentrate on separating GvHD and GvL in order to improve the clinical outcome of transplanted patients [85].

4. Impact of CD39 on Graft Rejection after Solid Organ Transplantation

CD39/NTPDase1 is a ubiquitously distributed acidic glycoprotein with a molecular mass of 70–100 kDa, which hydrolyzes ATP to ADP and subsequently to AMP [86–88] without substantial accumulation of ADP in the extracellular space [7]. CD39 was initially defined as a B-cell surface maturation marker [89]. Experimental studies provide evidence that it is expressed also on subpopulations of T cells, NK cells, macrophages, DCs, and platelets [90], as well as by vascular endothelial cells [90], human placenta, lung, skeletal muscle, kidney, and heart [86]. Also MSCs show abundant expression of CD39 [14, 91]. Expression of CD39 on different kinds of malignant neoplasms, such as chronic lymphocytic leukemia [92], colorectal [93], and pancreatic cancer [94], has been reported.

The abundant expression of CD39 on immune cells suggests its involvement in the regulation of inflammatory responses. By metabolizing extracellular ATP, CD39 modulates purinergic signalling via P2X and P2Y receptors. At the same time, the catalytic activity of this enzyme leads to production of 5′-AMP which can be hydrolyzed to adenosine via the action of CD73, prostatic acid phosphatase or ALP. Thromboregulation [4, 95], protection against ischemia and hypoxia [96–98], modulation of skin inflammation [99], inflammatory bowel disease [100, 101], and tumor-induced immune suppression [93, 102] are some of the physiological and pathological processes in which CD39 is involved.

The role of CD39 in transplantation was initially investigated in xenotransplantation models. In a first model, the impact of CD39 on cardiac xenotransplantation was tested [42]. Cardiac xenografts from $Cd39^{+/+}$ and $Cd39^{-/-}$ C57BL/6 $\times$ 129 Svj mice were transplanted into Lewis rats and rejection was diagnosed by cessation of ventricular contractions, as well as by direct visualization and histological examination. In certain cases, recipients were

additionally presensitized by injection of wildtype murine splenocytes seven days prior to transplantation, which led to HAR of the allograft. Alternatively, recipient animals were treated with cobra venom factor to achieve complement depletion, or treated with cyclosporine A. Interestingly, while CD39 mRNA levels increased 12 hours after transplantation, NTPDase enzymatic activity in the xenografts was reduced. In untreated recipients, presensitized recipients or recipients with complement depletion, there was no difference in the survival time between wildtype and CD39-deficient grafts. However, in a model with complement depletion in presensitized recipients, $Cd39^{-/-}$ grafts showed significantly reduced survival when compared to wildtype grafts. Additionally, in a model of long-term survival, CD39-deficient xenografts exhibited focal myocardial infarction as a result of increased intravascular platelet sequestration and fibrin deposition. In concordance with these data, in cardiac xenotransplantation with delayed xenograft rejection, CD39-deficient grafts showed reduced survival time and enhanced infarction, haemorrhage, and parenchymal destruction when compared to wildtype grafts [4]. Pathological features of the improved xenograft rejection included increased platelet aggregation, P-selectin expression, and endothelial cell activation. Collectively, these observations led to the hypothesis, that CD39 activity is required to maintain vascular integrity and inhibit platelet aggregation after transplantation. Other NTPDases seem to overtake at least in part the function of CD39 in genetically deficient grafts, as the basal NTPDase enzymatic activity was the same in $Cd39^{+/+}$ and $Cd39^{-/-}$ cardiac xenografts. This might be one possible explanation why CD39-deficient xenografts show in some models comparable survival time to that of wildtype xenografts [42].

The same authors performed investigations in another cardiac xenograft model, using Hartley guinea pigs as donors and Lewis rats as recipients [43]. Grafts were infected *in vitro* with recombinant adenoviruses containing human CD39 or β-galactosidase gene. As expected, infection with the CD39-containing adenovirus led to significantly prolonged xenograft survival with reduced vascular thrombosis. These results are in conformity with earlier observations that administration of a soluble apyrase derived from potatoes increases the survival of cardiac xenografts [51]. In concordance with these observations, administration of the soluble recombinant apyrase APT102 improved oxygenation and decreased lung pulmonary edema in a rat syngeneic lung transplantation model [52]. Additionally, apyrase treatment resulted in lower apoptosis rates in endothelial cells, attenuated proinflammatory cytokine expression and neutrophil sequestration.

Furthermore, transgenic mice expressing the human CD39 gene under the control of the H-2^b promoter were generated [44]. These mice had no increased spontaneous bleeding tendency under normal circumstances; they had normal platelet counts and coagulation parameters. However, the bleeding time in these mice was prolonged. They were subsequently used as donors in an allogeneic cardiac transplantation model and the survival of the allografts was compared to that of wildtype allografts after administration of anti-αGal IgG1 mAb to the $\alpha Gal^{-/-}$ recipients. As αGal is the major porcine epitope recognized by the human immune system, the application anti-αGal IgG1 mAb induces a reaction similar to HAR. Within the first 24 hours, 87% of the allografts which did not overexpress hCD39 were rejected, displaying widespread intravascular thrombosis, infiltration of platelets, and destruction of the cardiac ultrastructure, compared to only 15% of the hCD39-overexpressing allografts.

Recently, a role for CD39 has been suggested also in kidney transplantation [46]. In a syngeneic murine kidney transplant model, donor mice transgenic for human CD39 were generated. These mice were used to test the impact of CD39 on ischemia-reperfusion injury, one of the main causes for DGF in the clinic. After a 5-hour period of cold ischemia, the kidneys which overexpressed CD39 were transplanted into wildtype recipients. CD39-overexpressing isografts showed improved survival rates, reduced acute tubular necrosis, lower creatinine values, and less apoptosis when compared to wildtype isografts. Moreover, the same study showed that transgenic expression of human CD39 had a protective role also against warm ischemia-reperfusion injury, leading to improved creatinine and urea levels, reduced apoptosis and lower numbers of infiltrating $CD4^+$ T cells, macrophages, and neutrophils. Since ischemia-reperfusion injury is a major cause for DGF, application of soluble CD39 or AR agonists might be a successful approach to prevent organ damage in kidney and other solid organ transplantations [103]. Indeed, CD39 has been shown to be beneficial in distinct ischemia-reperfusion models. CD39 deficiency led to reduced survival in a model of intestinal ischemia, combined with increased vascular leakage, whereas administration of soluble apyrase improved the survival, preserved the mucosal integrity, and decreased PMN infiltration and intestinal haemorrhage [97]. In a model of ischemic preconditioning (IP) as protective mechanism in the case of ischemia-reperfusion injury, pharmacological blockade or genetic deletion of CD39 reversed the cardioprotection following IP [104]. This led to increased infarct sizes in $Cd39^{-/-}$ mice subjected to ischemia, while treatment with apyrase reduced infarct sizes. Similar observations were made by the same group in a model of renal IP [105]. Interestingly, both studies show a selective induction of CD39 expression after IP, which is not observed for NTPDase2, -3, and -8, suggesting that CD39 as the main NTPDase on the vasculature plays the major role for maintaining the barrier function of the endothelium.

However, degradation of extracellular nucleotides via CD39 seems to be important for the survival and function of a transplanted organ not only in the early phase after transplantation. CD39 enzymatic activity also seems to dampen immune responses like allograft rejection. CD39 and CD73 are both abundantly expressed on murine Tregs [10, 45]. These two enzymes give Tregs the ability to metabolize the proinflammatory danger signal ATP and release the anti-inflammatory mediator adenosine which appears to be an important part of their immunosuppressive machinery. In a model of skin allograft rejection, adoptive transfer of Tregs from CD39-deficient mice failed to prevent skin allograft rejection as successfully as the transfer of wildtype Tregs [45].

5. Impact of CD73 on Graft Rejection, Acute GvHD and GvL Effect

CD73 (ecto-5′-nucleotidase) is an ectonucleotidase which catalyzes the hydrolysis of extracellular nucleoside 5′-monophosphates to the respective nucleosides [34]. Thus CD73 is the crucial enzyme regulating the last degradation step of extracellular nucleotides. A variety of normal tissues express CD73, such as subsets of B and T lymphocytes [34], Tregs [45], MSCs [14], and also intestinal epithelial cells [106], endothelial cells of capillaries and venules, cells in the basal layer of nonkeratinizing squamous epithelium [36], retinal photoreceptor precursor cells [107] and the male murine reproductive tract [108]. Recent studies report expression of CD73 by different tumors, for example, in chronic lymphocytic leukemia [109], in ovarian [110], and breast cancer [111].

At least four functions of CD73 have been discussed in the literature [112]. First, CD73 generates nucleosides for the purine salvage pathway. This is followed by reuptake of the nucleosides via facilitated diffusion in the neighbour cells which use them to recover DNA and RNA bases and subsequently synthesize new nucleotides to meet critical metabolic needs of the cell [112]. Second, CD73 generates adenosine which activates the P1 purinergic (adenosine) receptors. ARs are seven-transmembrane domain G-protein-coupled receptors. The A_1 and A_3 receptors bind to a G_i protein and decrease the intracellular concentration of cAMP by inhibiting the adenylyl cyclase whereas the A_{2A} and A_{2B} receptors bind to a G_S protein and increase the intracellular concentration of cAMP by stimulating the adenylyl cyclase. The A_{2B} and A_3 receptors can additionally interact with a G_q protein and stimulate the phospholipase C [113]. However, signalling cascades of ARs are much more complex since they have been shown to modulate also protein kinase C, phospho-inositide 3 kinase, and mitogen-activated protein (MAP) kinases [114]. ARs have different affinity towards their substrate. While the half maximal effective concentration (EC_{50}) of the A_1, A_{2A}, and A_3 receptor is between 0.01 and 1 μM, activation of the A_{2B} receptor requires adenosine levels above 10 μM (EC_{50} 24 μM). This means that the A_{2B} receptor is not activated under physiological conditions but plays a role rather only when adenosine concentration is elevated due to cellular stress [113]. Depending on the receptor subtype tissue distribution, adenosine has pro- or anti-inflammatory properties. However, in the majority of clinically relevant models, adenosine serves as an anti-inflammatory signal and counteracts the proinflammatory reactions induced by the presence of ATP in the extracellular space.

Other functions discussed so far for CD73 are a co-receptor function in T-cell signalling and a role in cell adhesion [112]. Overexpression of CD73 in various tumor cell types, such as breast cancer cells [115] and glioma cells, [116] increases their adhesion capability and subsequently promotes migration and invasion. However, regulation of cell adhesion by CD73 seems to be a complex physiological process which depends on the particular cell type involved. Other experimental evidence shows that CD73 limits the expression of lymphocyte adhesion molecules on endothelial cells. Knockdown of CD73 on human umbilical vein endothelial cells (HUVECs) led to increased levels of ICAM-1, VCAM-1, and E-selectin mediated at least in part by activation of the transcription factor NF-κB [117].

CD73 is involved as immunomodulatory molecule in diverse models, such as acute lung injury [118, 119], chronic bleomycin-induced lung injury [120], gastritis [121], hepatic fibrosis [122], and sepsis [123]. Similar to CD39, it plays a protective role in hypoxia and ischemia-reperfusion injury. Intact CD73 expression was shown to be important for reducing the vascular leakage during hypoxia [124]. In this study, mice were subjected to normobaric hypoxia (8% O_2, 92% N_2) and increased vascular leakage was found in colon, liver, lung, muscle, heart, and kidney of $Cd73^{-/-}$ mice when compared to wildtype littermates. The same results were observed in mice treated with the specific inhibitor of CD73 enzymatic activity, adenosine-5′-(α, β-methylene)diphosphate (APCP), while administration of 5'-nucleotidase enzyme purified from *C. atrox* venom enhanced the vascular barrier function in CD73-deficient animals.

Other authors imply a role for CD73 and adenosine in cardiac and renal IP [125, 126]. In CD73-deficient mice and in mice treated with CD73 inhibitor, the protective effect of cardiac IP is reduced, leading to significantly increased infarct size and plasma levels of murine myocardial ischemia markers. Administration of 5′-nucleotidase enzyme leads to reconstitution of the wildtype phenotype and the A_{2B}-AR has been shown to be involved in the mediation of cardioprotection by CD73-generated adenosine. These data indicate that CD39 and CD73 act synergistically and play a crucial role to protect the endothelial barrier function in multiple organs under conditions of ischemia, hypoxia, and cell stress. In the context of transplantation, these findings suggests that pretreatment with soluble CD39 and CD73 enzyme, overexpression of these proteins or administration of an AR agonist might be a successful approach to reduce the rates of DGF as a common cause for graft failure in the early phase after transplantation.

CD73 and its product adenosine have also been implicated as regulatory mechanisms in allograft rejection in cardiac and tracheal transplantation. In a first model, the role of CD73 in a heterotopic murine cardiac transplantation model was tested. The authors focused on acute graft rejection and cardiac allograft vasculopathy, a rapidly progressive form of atherosclerosis which is the major cause of long-time failure of human cardiac allografts [47]. Here, CD73-deficiency of either donor or recipient led to significantly reduced allograft survival. In accordance with the protective role of CD73 in ischemia-reperfusion injury, permeability in cardiac allografts at four hours after transplantation was significantly increased in transplants with CD73-deficient donor or recipient. Additionally, increased infiltration with neutrophils and myeloperoxidase activity were observed. With respect to acute graft rejection, the authors found that CD73 deficiency led to greater cardiomyocyte damage, significantly higher parenchymal rejection scores and elevated numbers of infiltrating $CD4^+$, $CD8^+$, and $CD11b^+$ cells seven days after transplantation. At the same time

point, increased mRNA levels of cytokines (IL-1β, TNF-α, IFN-γ, and MCP-1) and adhesion molecules (ICAM-1 and VCAM-1) were detected in the case of CD73-deficient donor or recipient. These observations are compatible with earlier data which show involvement of adenosine in the suppression of proinflammatory cytokine production [127, 128]. Additionally, 60 days after transplantation, CD73-deficient allografts showed more severe luminal occlusion in the graft coronary arteries correlating to cardiac allograft vasculopathy, as well as significantly higher levels of donor-reactive alloantibodies in the chronic rejection phase. These effects were at least in part mediated via the A_{2B}-AR.

Furthermore, CD73-mediated adenosine production was suggested as a tolerogenic mechanism in trachea transplantation [48]. In this study, the authors used orthotopic murine trachea transplantation as a model for the development of bronchiolitis obliterans, one of the main long-term complications in human lung transplantation. This study showed that only CD73 deficiency of the recipient but not of the donor led to significantly increased graft luminal narrowing as an indicator of bronchiolitis obliterans. This was accompanied by 66% increase in the number of infiltrating $CD3^+$ T cells and significantly higher mRNA expression levels of IFN-γ and IL-2. These effects were mediated at least in part by the A_{2A}-AR, as treatment with the A_{2A} receptor agonist CGS-21680 led to reduced expression of proinflammatory cytokines and decreased the graft luminal narrowing as well as the number of infiltrating $CD3^+$ T cells.

Adenosine signalling is involved as an immunomodulatory pathway in some other models of allograft rejection. In a swine model of lung transplantation following ischemia-reperfusion injury, treatment with the A_{2A} receptor agonist ATL146e led to significantly lower lung injury score, decreased concentrations of serum TNF-α and neutrophil sequestration [129]. In a rat orthotopic model of small-for-size liver transplantation, administration of another A_{2A} receptor agonist, CGS21680, increased the allograft survival rate from 16.7% to 83.3%, and led additionally to improved liver function, preserved hepatic architecture, reduced neutrophil infiltration, and decreased secretion of TNF-α, IL-1β, and IL-6 [130]. These effects could be reversed by the simultaneous application of ZM241385, a selective A_{2A} receptor antagonist. Taken together, these studies suggest that activation of the A_{2A} receptor attenuates alloantigen responses [131].

CD73 regulates alloimmunity not only in solid organ transplantation but also in allo-HCT. Allo-HCT is performed as a treatment option for patients with hematologic malignancies more than 25 000 times worldwide per year [78]. One of the major complications limiting its success is the development of acute or chronic GvHD. We investigated the role of CD73 and endogenous adenosine in a model of murine acute GvHD with an MHC major mismatch between donor and recipient [49]. We observed that CD73 deficiency of donor or recipient led to significantly aggravated GvHD with reduced survival of the recipient, increased GvHD histopathology score and elevated concentrations of IL-6 and IFN-γ in the serum of recipient mice. Furthermore, genetical deletion of CD73 resulted in increased proliferation of alloreactive $CD4^+$ and $CD8^+$ T cells. These data are compatible with previous reports which show that even low concentrations of extracellular adenosine and AR agonists inhibit T-cell activation and expansion via binding to the A_{2A}-AR [132]. Interestingly, we found that endogenous adenosine binding to the A_{2A}-AR limits the expansion of alloreactive T cells and dampens the severity of acute GvHD. Our results extend previous reports [133] which suggest that activation of the A_{2A}-AR via the selective agonist ATL146e improves the survival of GvHD mice without affecting the donor cell engraftment. In this study, treatment of T cells with ATL146e reduced *in vitro* migration towards the chemokines CCL20, CXCL12, and CXCL10 by at least 30%, while *in vivo* administration of this substance decreased the serum levels of various proinflammatory cytokines. A_{2A}-AR activation also improved the clinical condition of mice with already established GvHD by reversing weight loss in these animals.

We investigated additionally the impact of CD73 on GvL activity in mice subjected to allo-HCT. As models of solid organ transplantation show that CD73 deficiency leads to more severe allograft rejection [47, 48], we hypothesized that pharmacological inhibition of this enzyme might improve the GvL effect. Mice underwent allo-HCT and were injected with malignant B cell lymphoma cells and treated either with the selective CD73 inhibitor, APCP, or with vehicle. Mice treated with APCP showed significantly reduced expansion of tumor cells as measured by bioluminescence imaging and improved survival when compared to the control group. Hence, we concluded that CD73 might have different roles after allo-HCT. On the one hand, patients developing acute GvHD might be treated with the soluble CD73 enzyme or with AR agonists to control this immunologic reaction, especially in the case of a benign underlying disease when GvL effect is not required. On the other hand, in patients with malignant diseases who receive DLI after transplantation, administration of a CD73 inhibitor might be a successful way to improve the GvL activity and prevent disease relapse.

The importance of CD73 in antitumor immunity has been studied intensively in the last years as well. CD73 expressed by tumor cells suppresses the host immune response and enhances migration, invasion, and metastasis in models of breast [111], and ovarian cancer [134], melanoma [135], colon carcinoma [111] and others. The role of CD73 in antitumor immunity and its potential implications for the clinic have been reviewed elsewhere [136, 137].

6. Clinical Implications for the Use of Ectonucleotidases as Modulators of Purinergic Signalling

Purinergic signalling is now one of the well established mediator pathways which play a key role in inflammation. Here, we discussed the beneficial effects of NTPDase1/CD39 and ecto-5′-nucleotidase/CD73 in solid organ transplantation and allo-HCT.

Both CD39 and CD73 have positive effects in the context of ischemia-reperfusion injury suggesting that they can reduce the rates of DGF. Despite strongly reduced ischemia length, reperfusion of newly transplanted organs still leads to an inflammatory response and postperfusion complications. Leukocytes migrating into the transplanted tissue release proinflammatory cytokines and free radicals which lead to direct tissue damage and attract further immune cells. CD39 and CD73 reduce vascular leakage by degrading extracellular ATP to adenosine. Indeed, it has been shown that elevated concentrations of extracellular ATP or UTP are associated with increased expression of the adhesion molecule VCAM-1 via the $P2Y_2$ receptor on endothelial cells [138]. Furthermore, ATP has been shown to increase the adherence of human PMN and the myeloid progenitor cell line HL-60 [139] and to modulate neutrophil recruitment to sites of sterile inflammation [140]. The latter appears to be a result from the activation of the NLRP3 inflammasome via the P2X7 receptor on macrophages. Activation of the NLRP3 inflammasome leads to enzymatic cleavage and release of IL-1β and IL-18. Neutrophils are then attracted to these sites of sterile inflammation due to increased concentration of chemotactic signals and can exacerbate dramatically local tissue damage. On the other hand, adenosine reduces the expression of E-selectin and VCAM-1 as well as the production of IL-6 and IL-8 [141]. Additionally, previous reports suggest that, treatment with adenosine decreases neutrophil adhesion in an *in vitro* ischemia-reperfusion model [142] and PMN-mediated adenosine release diminishes endothelial paracellular permeability via the activation of the A_{2B} receptor [143]. Taken together, these data indicate that CD39 and CD73 metabolize extracellular ATP, which serves as a danger signal and promotes tissue injury after reperfusion, and further lead to release of adenosine, which decreases the secretion of proinflammatory cytokines and the adherence of PMN to the endothelium. This helps maintain the barrier function of the endothelium under cell stress conditions, so that application of soluble forms of CD39 and CD73 might reduce ischemia-reperfusion injury and DGF in the clinic.

CD39 has been extensively studied in xenograft rejection. Xenotransplantation has been widely discussed as a possible solution for the lack of donor organs and the long waiting time on transplant lists. The success of this therapeutic modality has been limited mostly by HAR. HAR is induced by preformed antibodies against certain antigens like αGal. In 2004, transgenic swine lacking the gene for α-1,3-galacotosyltransferase were generated [58]. This led to significantly prolonged survival of transgenic hearts transplanted in baboons [59]. CD39 is another protective mechanism for xenografts due to its ability to maintain vascular integrity and inhibit platelet aggregation. Indeed, CD39 degrades ATP as well as ADP and decrease of the extracellular ADP concentration inhibits platelet aggregation. Mice overexpressing human CD39 have increased bleeding times and their platelets show attenuated initial response to collagen and ADP. Interestingly, transgenic mice are also resistant to systemically induced thromboembolism [44]. Wildtype mice, injected intravenously with collagen and ADP, suffered to 90% from cardiorespiratory arrest and immediate death, whereas in the group of transgenic mice only 7% died. The response to either only collagen or only ADP was also attenuated in CD39-overexpressing mice. These data have implications for the clinic, as treatment with apyrase, a soluble form of CD39, might prevent thrombosis as one of the critical mechanisms mediating HAR.

Furthermore, CD39 and CD73 modulate the severity of acute allograft rejection and acute GvHD. There are at least three possible ways in which ectonucleotidases can influence allorecognition: (i) release of adenosine in the proinflammatory microenvironment by resident endothelial cells, (ii) production of adenosine by Tregs as one of their immunosuppressive mechanisms, (iii) generation of adenosine by MSCs which are also known to induce long-time allograft tolerance.

Adenosine is a potent inhibitor of T-cell activation. As CD39 and CD73 are expressed on endothelial cells, adenosine is generated within the inflammatory microenvironment after transplantation and can exert direct effects on alloreactive T cells as well as on other immune cells. AR signalling decreases the proinflammatory cytokine production and the proliferation of T cells [132, 144] and attenuates the alloantigen presenting properties of DCs [145]. Effector T cells express A_{2A} [146] and A_{2B}-ARs [147] which are G_s-protein-coupled and increase intracellular cAMP levels. This, in turn, leads to inhibition of TNF-α and IFN-γ production and reduces T-cell activation in ConA-induced liver damage, chemically induced hepatotoxicity and septic shock model after LPS injection [146, 148]. Additionally, adenosine regulates innate immune cell activity, preventing tissue damage caused by PMN and macrophages [149]. Interestingly, adenosine has direct effects on endothelial cells as well. CD73 depletion induces an upregulation of the adhesion molecules ICAM-1, VCAM-1, and E-selectin on HUVECs [117] and might thus enhance lymphocyte transmigration. CD73 deficiency also leads to cell elongation and actin stress fibre formation in HUVECs, indicating again an important role for adenosine signalling in regulating endothelial cell permeability. However, adenosine can be generated not only by the resident endothelial cells but also by Tregs and MSCs. Adenosine production via CD39 and CD73 expression is one of the immunosuppressive pathways by which murine Tregs modulate the activity of other immune cells. Tregs are characterized by the expression of the transcription factor Foxp3 and the α-chain of the IL-2 receptor (CD25) [150]. In animal models of solid organ transplantation and allo-HCT, Treg infusion protects skin and cardiac allografts [151] and prevents successfully acute GvHD [152]. Furthermore, *ex vivo* expanded Tregs have the ability to suppress skin allograft rejection and transplant arteriosclerosis [153, 154]. Tregs inhibit T cell activation via direct cell-to-cell contact and secretion of IL-10 and TGFβ, leading to inhibition of intranuclear gene transcription [150]. It is now well established that CD39 and CD73 are expressed on murine Tregs and that adenosine production is necessary for proper Treg function [10, 45]. Taken together, these data imply that intact CD39 and CD73 expression on Tregs might be one further important mechanism which

Table 1: Impact of ectonucleotidases on solid organ transplantation and allo-HCT.

Model	Ectonucleotidase	Biological impact	Reference
Cardiac xenograft transplantation	CD39	Attenuated survival of CD39-deficient xenografts in a model of delayed xenograft rejection with enhanced parenchymal injury, infarction and platelet aggregation	[4]
Cardiac xenograft transplantation	CD39	Increased intravascular platelet sequestration and focal myocardial infarction in complement-depleted, presensitized $Cd39^{-/-}$ recipients	[42]
Cardiac xenograft transplantation	CD39	Adenovirus-mediated CD39 overexpression leads to significantly prolonged xenograft survival with reduced vascular thrombosis	[43]
Cardiac allograft/discordant xenograft transplantation	CD39	Attenuated platelet deposition with preserved cardiac architecture and improved graft survival in mice overexpressing hCD39	[44]
Murine allogeneic skin transplantation with adoptive Treg transfer	CD39	CD39-deficient Tregs fail to suppress skin allograft rejection	[45]
Murine syngeneic kidney transplantation	CD39	Reduced acute tubular necrosis and apoptosis, improved graft function and prolonged survival in hCD39 overexpressing isografts	[46]
Murine allogeneic cardiac transplantation	CD73	Reduced graft survival and more severe cardiac allograft vasculopathy when donor or recipient is CD73-deficient	[47]
Murine allogeneic tracheal transplantation	CD73	$Cd73^{-/-}$ recipients show significantly reduced allograft survival with increased airway luminal obliteration and T-cell infiltration	[48]
Murine allogeneic hematopoietic cell transplantation	CD73	CD73 deficiency of donor or recipient enhances acute GvHD severity and pharmacologic CD73 blockade improves GvL activity	[49]
Murine allogeneic hematopoietic cell transplantation	Apyrase treatment	Reduced acute GvHD severity, T cell expansion, IFN-γ production and increased Treg numbers	[50]
Cardiac xenograft transplantation	Apyrase treatment	Attenuated intragraft platelet aggregation and prolonged survival time	[51]
Rat syngeneic lung transplantation	Apyrase treatment	Protection against pulmonary edema, improved oxygenation, attenuated neutrophil activity, apoptosis, and inflammatory cytokine production	[52]

lead to Treg-mediated allograft tolerance and reduced GvHD severity. Despite the strong expression of CD39 and CD73 by murine Tregs, only 47% of the human Tregs have been found to express both ectonucleotidases [121]. These data suggest that, in the human setting, the impact of adenosine generation as an inhibitory mediator released by Tregs might not be as substantial as in the murine preclinical models. The role of ATP metabolization by Tregs in transplantation has been reviewed elsewhere [155].

The third cell population which has the capacity to generate extracellular adenosine is MSCs. MSCs are multi potent progenitor cells which have the capacity to differentiate into mesoderm and nonmesoderm-derived tissues like chondrocytes, osteocytes, myocytes, hepatocytes, adipocytes and neuron-like cells [156, 157]. They were initially described in the bone marrow but have a rather broad tissue distribution and can be isolated also from umbilical cord blood, adipose tissue, placenta, periosteum, trabecular bone, synovium, skeletal muscle, and deciduous teeth [157]. Well-known functions of MSCs include maintenance of the hematopoietic stem cell niche, wound healing, and organ regeneration [156]. In the past years, MSCs have emerged as one of the key cell populations which regulate inflammation and autoimmune diseases. Moreover, they have been implied in solid organ transplantation and allo-HCT. MSCs express a variety of cell surface molecules, including CD39 [14, 91] and CD73 [14]. Indeed, CD73 is one of the markers proposed to distinguish hematopoietic stem cells from MSCs [156]. MSCs have the capacity to suppress allospecific T cell proliferation and to reduce the production of TNF-α and IFN-γ *in vitro*. Additionally, in an *in vivo* model of kidney transplantation after prolonged cold ischemia, MSC injection decreased the expression of proinflammatory cytokines and the infiltration of macrophages and DCs into the allograft [158]. Moreover, MSCs impair DC-activation via TLR4, inducing decreased expression of CD40, CD80, CD86, MHC I and MHC II, and TNF-α secretion. Additionally, MSC-conditioned DCs showed reduced ability to prime $CD4^+$ T cells and to activate $CD8^+$ T cells [159]. *In vivo* studies showed that MSC infusion prolonged the survival of kidney allografts by preventing acute cellular rejection [160]. There is evidence that MSCs have beneficial effects also in models of liver [161, 162], heart [163, 164], and skin [165] transplantation. Generation of adenosine by CD39 and

CD73 is one of the potential mechanisms by which MSCs might regulate allograft rejection. Interestingly, MSCs up regulate CD39 and increase adenosine production in order to suppress the activation of T cells [14, 159]. Treatment with POM-1, a selective NTPDase-inhibitor, or an A_{2A}-AR antagonist abolished the immunosuppressive effect of MSCs on T cells in both human and murine models [14, 159]. These data provide evidence that MSCs suppress the activation of T cells and reduce the production of proinflammatory cytokines as one of the possible mechanisms by which they enhance allograft tolerance.

Similar results have been obtained after injection of MSCs in allo-HCT recipients. Clinical studies with patients suffering from steroid-refractory GvHD showed that repeated MSC infusions can treat severe GvHD [166, 167] and animal studies showed a dose-dependent inhibition of GvHD development by MSCs [168]. Since endogenous adenosine [49] as well as treatment with an adenosine receptor agonist [133] reduces the severity of acute GvHD, it is possible that namely adenosine mediates the observed effects of MSCs after allo-HCT.

Notably, in a model of allogeneic liver transplantation, MSC-mediated protection was connected to increased expansion of Tregs [161]. Other studies also prove the capacity of MSCs to induce differentiation of T cells into Tregs [169, 170]. These data provide a possible link between the function of these two cell populations in adenosine production and suppression of alloreactivity.

Degradation of extracellular ATP and production of adenosine might be enhanced by administration of a soluble form of CD39/apyrase and of CD73. Soluble CD39 has been successfully purified from High Five insect cells [171] and isolated as a recombinant enzyme from COS-1 or Chinese hamster ovary cell lines [172]. Apyrase can also be derived from potatoes [173]. Soluble CD73 has been isolated from *Crotalus atrox* venom. Human and murine recombinant ecto-5′-nucleotidase have also been purified [174, 175]. These sources might be relevant for conduction of animal or clinical studies on the effect of ectonucleotidases *in vivo* in solid organ transplantation and allo-HCT.

Adenosine is the final product of ectonucleotidase activity, so that modulating AR activity might be an alternative way to exploit purinergic signalling in the clinic. There are at least 15 AR agonists and more than 20 AR antagonists. Regadenoson, the first FDA-approved A_{2A}-AR agonist, can be administered as a potent coronary vasodilator in the clinic. However, the biological half-life of regadenoson is only about 2-3 minutes; adenosine itself has a half-life of less than a minute. Selective AR agonists with a longer half-life would be required for treatment of allograft rejection and GvHD. As ARs are ubiquitously expressed, possible side effects on cardiac and pulmonary function should be taken into careful consideration.

7. Conclusions

Purinergic signalling modulates the severity of ischemia-reperfusion injury, alloantigen recognition, graft rejection, acute GvHD, and GvL activity through pleiotropic mechanisms (Table 1). It has been shown that two major ectonucleotidases, CD39 and CD73, regulate these responses by metabolizing the proinflammatory ATP to the anti-inflammatory product adenosine. Important cell populations expressing CD39 and CD73 include endothelial cells, Tregs, and MSCs. These cell populations function synergistically to maintain the physiological balance between nucleotides and nucleosides in the extracellular space. Endogenous adenosine and exogenous AR agonists modulate ischemia-reperfusion injury and suppress alloimmune responses by reducing the proliferation and cytokine secretion of T cells, as well as the antigen-presenting capacity of DCs. These data suggest potential clinical applications of soluble ectonucleotidases and AR agonists/antagonists for regulation of the strength of alloimmune responses which can be tailored according to the clinical situation.

Abbreviations

5′-AMP:	5′-adenosine monophosphate
ADP:	Adenosine diphosphate
Allo-HCT:	Allogeneic hematopoietic cell transplantation
ALP:	Alkaline phosphatase
AMP:	Adenosine monophosphate
AMR:	Antibody-mediated rejection
APC:	Antigen-presenting cell
APCP:	Adenosine 5′-(α,β-methylene)diphosphate
AR:	Adenosine receptor
ATP:	Adenosine triphosphate
cAMP:	Cyclic adenosine monophosphate
DAMP:	Danger-associated molecular pattern
DC:	Dendritic cell
DGF:	Delayed graft function
DLI:	Donor lymphocyte infusions
EC:	Effective concentration
GIT:	Gastrointestinal tract
GvHD:	Graft-versus-host disease
GvL:	Graft-versus-leukemia
HAR:	Hyperacute rejection
HLA:	Human leukocyte antigen
HUVEC:	Human umbilical vein endothelial cell
IP:	Ischemic preconditioning
LPS:	Lipopolysaccharide
MAP:	Mitogen activated protein
MHC:	Major histocompatibility complex
MSC:	Mesenchymal stromal/stem cell
NDP:	Nucleoside diphosphate
NK cell:	Natural killer cell
NPP:	Nucleotide pyrophosphatase/phosphodiesterase
NTP:	Nucleoside triphosphate
NTPDase:	Nucleoside triphosphate diphosphohydrolase
PAMP:	Pathogen-associated molecular pattern
PAP:	Prostatic acid phosphatase

PMN: Polymorphonuclear leukocytes
PRR: Pattern-recognition receptor
TLR: Toll-like receptor
Treg: Regulatory T cell
UDP: Uridine diphosphate
UTP: Uridine triphosphate
αGal: Galactose-α(1,3)-Galactose.

Conflict of Interests

The authors have no competing financial interests to declare.

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