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Unlocking the Potential of Purinergic Signaling in Transplantation

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Short title: Purinergic Signaling in Transplantation

Abbreviations:

ADP, adenosine diphosphate

allo-HSCT, Allogeneic hematopoietic stem cell transplantation

AMP, adenosine monophosphate

APC, antigen-presenting cell

AT1R, angiotensin type 1 receptor

ATP, adenosine triphosphate

CD40L, CD40 ligand

CNI, calcineurin inhibitor

eATP, extracellular ATP

GvHD, graft-versus-host disease

IRI, ischemia-reperfusion injury

iTreg, CD4⁺ CD25^{high} CD127^{low} FoxP3⁺ regulatory T cell

MMF, Mycophenolate mofetil

MPA, mycophenolic acid

MSC, mesenchymal stromal/stem cell

mTOR, mammalian target of rapamycin

NFAT, nuclear factor of activated T cells

NPP, nucleotide pyrophosphatase/phosphodiesterase

oATP, oxidized ATP

PAR, protease-activated receptors

T-reg, T regulatory cell

Abstract:

Purinergic signaling has been recognized to play an important role in inflammation, angiogenesis, malignancy, diabetes and neural transmission. Activation of signaling pathways downstream of purinergic receptors may also be

implicated in transplantation and related vascular injury. Following transplantation, the pro-inflammatory “danger signal” adenosine triphosphate (ATP) is released from damaged cells and promotes proliferation and activation of a variety of immune cells. Targeting purinergic signaling pathways may promote immunosuppression and ameliorate inflammation. Under pathophysiological conditions, nucleotide-scavenging ectonucleotidases, CD39 and CD73, hydrolyze ATP, ultimately to the anti-inflammatory mediator adenosine. Adenosine suppresses pro-inflammatory cytokine production and is associated with improved graft survival and decreased severity of graft-versus-host disease. Furthermore, purinergic signaling is both directly and indirectly involved in the mechanism of action of several existing immunosuppressive drugs, such as calcineurin inhibitors and mTOR inhibitors. Targeting of purinergic receptor pathways, in particular in the setting of combination therapies, could become a valuable immunosuppressive strategy in transplantation. This review article focuses on the role of the purinergic signaling pathway in transplantation, immunosuppression, and explores possible future applications in clinical practice.

Introduction

Organ, tissue and hematopoietic cell transplantation are often end-stage treatment strategies considered as salvage therapy. In the past decade, increasing public awareness, and the subsequent increase in the number of donors, has resulted in higher transplant frequencies with generally excellent outcomes.(reviewed in 1) However, the long-term success of solid organ and hematopoietic cell transplantation, which is characterized by unfettered inflammation and immunological complications, associated with ischemia-reperfusion injury (IRI), delayed graft function or altered engraftment, rejection, and graft-versus-host disease (GvHD), remains a major challenge. Transplantation-related metabolic changes may be associated with the activation of the purinergic signaling pathway, which has important metabolic and regulatory roles in multiple areas, such as inflammation, angiogenesis, malignancy, diabetes, and neural transmissions.(2, reviewed in 3, 4-6) Following transplantation, damaged/ischemic cells release the nucleotide adenosine triphosphate (ATP), which promotes inflammation, T-cell activation, and proliferation of immune cells leading to further cell damage.

The purinergic signaling pathway has been covered in detail in many reviews over the past decade(reviewed in 7) but briefly it involves the binding of extracellular ATP (eATP) to ion channel P2X receptors (P2X1-7) and G-protein-coupled P2Y receptors (P2Y1, 2, 4, 6, 11-14); the distribution of receptor subtypes and their selective agonists and antagonists are summarized in Table 1. Receptor binding, either in an autocrine or paracrine fashion, promotes the proliferation of immune cells, T-cell activation, and inflammation.(reviewed in 8, 9, 10) Therefore, targeting the purinergic signaling pathway to alleviate inflammation and promote immunosuppression offers a promising experimental strategy for improved outcomes in both solid organ grafting and hematopoietic stem cell transplantation. One such target is the ectonucleotidases (CD39 and CD73) which hydrolyze eATP to adenosine diphosphate (ADP), and subsequently to adenosine monophosphate (AMP) and adenosine. ADP can activate P2Y receptors,(reviewed in 9, 11, 12) while AMP and adenosine bind G-protein-coupled P1 receptors (P1A1, 2a, 2b, 3) to promote T regulatory (T-reg) cell proliferation and immunosuppression(reviewed in 8, 9) (Figure 1).

In 2011, approximately 170,000 individuals in the US were living with a transplanted organ, the majority of whom require continual immunosuppressive therapies to maintain tolerance and prevent organ rejection.(reviewed in 13) Existing immunosuppressive regimens impact the regulation of purinergic responses at the level of agonist/mediator release, receptor functionality, and the nucleotide-scavenging ectonucleotidases that ultimately generate adenosine.(reviewed in 14) Current immunosuppressive drugs, such as mammalian target of rapamycin (mTOR) inhibitors, mycophenolic acid (MPA), or calcineurin inhibitors (CNIs), are closely linked to cell metabolism and might affect, while simultaneously being impacted by, purinergic signals. The purpose of this review is to explore the role of purinergic signaling in transplantation and immunosuppression, and the implications for clinical practice.

Purinergic signaling in specific organs, tissues and cells

Liver

Purinergic signaling is implicated in a variety of liver diseases and post-transplantation outcomes such as inflammation, rejection, hepatic IRI, hepatic regeneration, steatohepatitis, fibrosis, and cancer.(15-18, reviewed in 19, 20) The liver produces the majority of the nucleotides in the body, and concurrently has high ATPase and ADPase activities. Mechanical stress during transplantation results in the secretion of ATP or ADP from hepatocytes and Kupffer cells, which subsequently bind P2X and P2Y receptors expressed on hepatocytes, hepatic sinusoids, and other resident cells, or bind P1 receptors following hydrolysis to adenosine by select ectonucleotidases (Figure 2).(reviewed in 21) Although adenosine can promote immunosuppression through the proliferation and activation of T-reg cells via activation of the P1A2a receptor,(reviewed in 22) protracted ectonucleotidase-dependent adenosine production following ethanol or fructose ingestion can lead to hepatic steatosis, fibrosis, and ultimately to cirrhosis. These diverse biological effects of adenosine have important implications for the development of purinergic therapies in liver transplantation.(reviewed in 21) CD39 is important for rapid post-transplant hepatic regeneration and subsequent graft outcomes, as evidenced by the reduced regenerative capacity of liver transplanted into CD39-deficient mice.(reviewed in 19). Transgenic CD39 has also been shown to be beneficial in protecting liver grafts from early IRI in mice after 18 hours of cold ischemia, an effect attributed to the depletion of hepatic CD4⁺ T cells, but not natural killer (NK)-T cells that may have a greater role in warm IRI.(23) Monitoring hepatic ATP levels in conjunction with serum hyaluronic acid has been suggested as an approach to assess graft viability and post-transplant instability.(reviewed in 24) The ImmuKnow™ assay (Cylex, Inc., Columbia, MD, USA) which measures peripheral blood CD4⁺ total ATP levels has shown promise in determining immunosuppression and diagnosing early rejection.(reviewed in 21)

Kidney

Purinergic P2X and P2Y receptors are expressed on a variety of renal cells, including mesangial cells, podocytes and cells of the parietal sheets (Figure 2), and all renal cells can produce ATP under conditions such as mechanical or osmotic stress, fluid flow or agonist stimulation.(reviewed in 25, 26) Furthermore, overexpression of the ectonucleotidase CD39 protected against renal IRI in a mouse model, raising the possibility that soluble CD39 (e.g. apyrase) could be used therapeutically to prevent organ damage in kidney transplantation.(8, reviewed in 27)

In a renal transplantation model characterized by 5 hours of cold preservation, mice receiving a CD39-overexpressing graft had better renal function, and remained healthy for a longer duration, compared with the control recipients. This effect was attributed to the increased production of adenosine in these mice, and the P1A2a receptor may also be involved, as blocking this receptor with a specific antagonist reversed the observed benefits.(28) In the same study, transgenic expression of human CD39 conferred renal protection in a mouse model of warm IRI, and together with results from the transplantation model, the authors concluded that CD39 expression had beneficial effects on both warm and cold renal IRI. (28)

Other studies have shown that P1A2a receptor activation considerably reduces T-cell mediated renal injury.(reviewed in 22) The P1A2b receptor was shown to protect against tissue injury and improve kidney function in a mouse model of renal ischemia using ischemic preconditioning, an effect attributed to the inhibition of neutrophil-dependent production of TNF- α .(29, 30) A recent review by Solini *et al.* gives a more detailed overview of the involvement of ATP and P2 receptors in both physiologic and pathologic signaling in the kidney.(reviewed in 31)

Heart

Some of the earliest work on the extracellular action of purines was performed on heart and blood vessels,(32, reviewed in 33, 34) and it is known that cardiac cells express purinergic receptors and ectonucleotidases (Figure 2).(reviewed in 22) An *in vitro* cardiac cell ischemia model showed that adenosine prevented necrosis and promoted cardioprotection through P1A2a/2b receptor activation.(35) Furthermore, adenosine-treated grafts in a rat heterotopic heart transplant model showed less inflammation and fewer infiltrating cells, with reduced subsequent IRI and myocardial injury compared with the control grafts.(36) This effect suggests that storing and transporting grafts in the presence of adenosine – as in UW solution – may reduce the impact of IRI, and improve graft outcomes.(reviewed in 22) Furthermore, cardiac xenograft rejection and vascular thrombosis observed in mice was reversed when CD39 was introduced either by adenovirus-mediated overexpression(37) or administration of apyrase.(38) Similarly, CD39 has been shown to be cardioprotective by reducing myocardial infarction following P1A2b-mediated coronary artery occlusion.(22) Studies in a cardiac allotransplantation model have demonstrated that reduced graft expression of P1A2b increased inflammatory and immune responses, and ultimately reduced allograft survival, when either the donor or recipient was CD73-deficient, demonstrating the protective effect of CD73.(39)

Targeting of the P2X7R receptor with oxidized ATP (oATP) was shown to promote cardiac transplant survival in 80% of murine recipients of a fully mismatched allograft, an effect that correlated with the inhibition of T-cell activation and differentiation (40).

Lung

Pulmonary and alveolar epithelial cells express purinergic receptors and ectonucleotidases (Figure 2),(reviewed in 22, 41, 42) and purinergic signaling is known to contribute to the pathophysiology of asthma and chronic obstructive pulmonary disease.(reviewed in 43) Inhibition of P2X7 signaling through the use of oxidized ATP, a specific P2X7

inhibitor, resulted in fewer inflammatory cells, less rejection, improved lung function, and prolonged survival of lung allografts in mice.(44) The suppression of inflammation by CD73-mediated adenosine production, particularly through the P1A2a receptor, is known to be crucial in graft survival and lung function.(reviewed in 22) In a porcine model of lung transplantation, activation of the P1A2a receptor during early reperfusion limited inflammation and preserved lung function.(45) Conversely, it has been suggested that activation of the P1A2b receptor may promote bronchiolitis obliterans syndrome, a manifestation of chronic pulmonary allograft dysfunction.(46) These findings suggest that a P1A2a receptor agonist, possibly coupled with a P1A2b receptor antagonist, might be beneficial in limiting acute pulmonary IRI, and subsequently improving graft survival and lung function following transplantation.

Pancreatic islets

Expression of purinergic receptors on islet beta cells has been documented (Figure 2), and plays a definitive role in insulin secretion.(reviewed in 47, 48) whereby elevated extracellular ATP levels have been associated with hyperinsulinemia.(49) A recent study by Vergani and colleagues reported the expression of P2X7 receptors on islets, and subsequent inhibition of this purinergic receptor using oxidized ATP in mouse models of islet allograft rejection.(50) This intervention resulted in a significant improvement in graft survival, with 30% of the mice maintaining good graft function at 100 days. Furthermore, when combined with sirolimus, the intervention resulted in favorable long-term graft outcomes at 100 days in 7 of the 10 mice. In another transplantation model, human CD39 overexpression in transgenic islets significantly delayed clot formation time in the presence of human blood, thus prolonging graft islet viability.(51)

The immunosuppressive effect of adenosine in inhibiting IFN- γ production from neutrophils has been shown to prevent early loss of transplanted islets in the liver of mice.(52) Another study using a multiple low-dose streptozotocin mouse model reported that overexpression of CD39, which promotes the production of adenosine, protected islet grafts from T cell-mediated injury and reduced susceptibility to diabetes.(53)

Endothelium

Extracellular ATP is rapidly broken down by ectoenzymes,(54) such as the nucleoside triphosphate diphosphohydrolase family, including NTPDase 1 (CD39), the nucleotide pyrophosphatase/phosphodiesterase (NPP) family, ecto-5'-nucleotidase (CD73), and alkaline phosphatase.(reviewed in 9, 55, 56, 57) CD39 is expressed on quiescent endothelial cells playing a critical role in thromboregulation,(58) while NTPDase-2 is widely expressed on vascular adventitial cells (Figure 2).(59)

In episodes of chronic rejection, the endothelial layer is invariably damaged, triggering a collateral pathway of coagulation mediated primarily through thrombin, a potent platelet agonist. The downstream effects of thrombin are mediated through protease-activated receptors (PARs),(reviewed in 60) which lead to the activation of platelets, endothelial cells, and a wide array of immune cells. This response is synergistically augmented through the release of ADP by activated platelets and endothelial cells from dense intracellular granules, resulting in ectonucleotidase-mediated production of AMP and adenosine.

Bearing in mind the destructive effects of thrombus formation in mediating transplant rejection and its biological pathways of formation, one can appreciate the role of ectonucleotidases in modulating allograft responses. Indeed, the activity of CD39 has been reported to be significantly decreased in chronic rejecting grafts, highlighting a potential therapeutic avenue of complementation. The viability of this prospect was confirmed in mouse models of rejection in conjunction with transgenic expression of human CD39 (hCD39). In this study, mice overexpressing hCD39 had increased cardiac allograft survival, along with protection from thrombosis, as compared with wild type mice, serving only to further highlight the previously untapped anti-thrombotic therapeutic potential of ectonucleotidases in transplantation.(61)

Stem cells – mesenchymal and hematopoietic

Allogeneic hematopoietic stem cell transplantation (allo-HSCT), a curative therapeutic option for patients with hematological cancers, is performed more than 27,000 times globally per year (CIBMTR report 2014).(62) However, GvHD remains a major obstacle for a broader application of allo-HSCT. Extracellular nucleotides and ectonucleotidases are involved in inflammation(reviewed in 12) and can be particularly implicated in GvHD.(63, 64) Interestingly, multiple studies show that mesenchymal stromal/stem cells (MSCs), which have the potential to upregulate CD39, CD73 (Figure 2) and increase adenosine production to suppress activated T cells,(65) promote tolerance following allo-HSCT,(66-70) diabetes,(71) and solid organ transplantation.(72) A recent publication has shown that suppressive-type MSC can be induced by IL-17A and promote the generation of CD4⁺ CD25^{high} CD127^{low} FoxP3⁺ regulatory T cells (iTregs) from activated CD4⁺ CD25⁻ T cells *in vitro*. These induced iTregs express both CD39 and CD73, as well as CD69, OX40, cytotoxic T-lymphocyte associated antigen-4, and glucocorticoid-induced TNF receptor-related protein. The generated, suppressive MSCs-17 are therefore able to boost iTreg functionality to potently suppress T cell activation, and appear to have minimal immunogenicity. These MSCs and other regulatory cells might be considered a potential option for patients with corticosteroid-refractory GvHD.(73)

Impact of purinergic signaling on immunosuppressive drugs

CNIs

The serine-threonine phosphatase calcineurin has a role in the activation of T cells, and inhibition of this protein by CNIs is a widely used treatment strategy to promote immunosuppression following transplantation. Cyclosporine forms a complex with the cytosolic protein cyclophilin, which binds and inactivates calcineurin, and ultimately inhibits the transcription of interleukin (IL)-2 and other pro-inflammatory cytokines by preventing calcineurin-dependent dephosphorylation of the transcription factor nuclear factor of activated T cells (NFAT) (Figure 3).(74, reviewed in 75) Tacrolimus, also known as FK506, inhibits NFAT dephosphorylation by calcineurin, although the precise mechanism of action differs from that of cyclosporine. Tacrolimus binds to the immunophilin FKBP12, forming a FKBP12-FK506 complex that inactivates calcineurin.(74, reviewed in 75)

ATP signaling through the P2Y2 receptor dose-dependently increased NFAT activity and intracellular Ca²⁺ in rat neonatal cardiac fibroblasts, with an associated decrease in angiotensin type 1 receptor (AT1R). This effect of ATP

was inhibited by the addition of cyclosporine, indicating that ATP-mediated NFAT induction is achieved through the calcineurin-NFAT pathway and is therefore inhibited by CNIs.(76) Inhibition of the P1A3 adenosine receptor with theophylline improved glomerular filtration rate and renal blood flow in an acute tacrolimus toxicity model in rats, suggesting a role for pathological levels of adenosine in nephropathies associated with CNI treatment.(77)

MPA

Mycophenolate mofetil (MMF) is an antiproliferative agent that is metabolized to MPA and inhibits the synthesis of guanine monophosphate, thereby blocking the proliferation of B- and T-lymphocytes. MPA achieves this effect by binding and inhibiting the enzyme inosine monophosphate dehydrogenase, the crucial enzyme of *de novo* purine synthesis (Figure 3).(reviewed in 78, 79) Due to this mechanism of action, MPA has a direct inhibitory effect on the synthesis of purines and can therefore modify the amount of eATP that is available for purinergic signaling. Indeed, MPA treatment in a primary human T-lymphocyte model resulted in up to 50% reduction in ATP concentration in comparison to control cells.(80)

mTOR inhibitors

The mTOR inhibitors, sirolimus and everolimus, block the activity of mTOR, a serine threonine protein kinase with multifarious physiological roles in regulating cell metabolism, growth, proliferation, angiogenesis, and immune responses. Both sirolimus and everolimus are similar to tacrolimus, in that they bind to the immunophilin FKBP12; however, rather than inhibiting calcineurin, the FKBP12-sirolimus or FKBP12-everolimus complex inhibits the activity of mTOR (Figure 3).(reviewed in 81)

The relative levels of ATP and adenosine in the extracellular space can directly influence the activation or inhibition of mTOR, and the subsequent induction of either pro-inflammatory or anti-inflammatory responses, respectively.(reviewed in 82) mTOR is known to be an important downstream effector protein in purinergic signaling, and mTOR phosphorylation is a required step in ATP-induced cell proliferation which can be inhibited by rapamycin/sirolimus and other mTOR inhibitors (e.g. AZD8055) that compete with ATP at the mTOR active site.(83, reviewed in 84) Treatment of mice with rapamycin/sirolimus in combination with oATP, an irreversible P2X7 purinergic receptor inhibitor, resulted in long-term islet function in 80% of transplanted mice, suggesting a synergistic effect between mTOR inhibitors and purinergic inhibitors that might be beneficial following transplantation in humans.(50)

When combined with the CNI cyclosporin, sirolimus treatment enhanced cyclosporine-induced inhibition of purinergic metabolism compared with cyclosporine alone in perfused rat brain slices after 4 hours of perfusion, suggesting a synergistic immunosuppressive activity between the CNI and the mTOR inhibitor.(85) However, this synergistic effect was not observed following similar combination treatment with cyclosporin and everolimus. Increasing blood concentrations of sirolimus decreased relative Krebs cycle urine metabolite levels in rats, but increasing everolimus concentrations did not have the same effect, either alone or in combination with cyclosporin.(86)

Other immunosuppressants

The anti-CD25 monoclonal antibody, basiliximab, binds to the IL-2 receptor alpha chain (Figure 3), thereby inhibiting purinergic signaling and other pathways leading to IL-2-mediated proliferation of T cells and allograft rejection.(reviewed in 87) The anti-CD40 monoclonal antibody lucatumumab acts by preventing the interaction between CD40 and CD40 ligand (CD40L), thus inhibiting the presentation of antigen to T cells. Interestingly, low concentrations of eATP have been shown to also interfere with the same target as lucatumumab, the CD40-CD40L complex, and subsequently inhibit the activation of T cells by dendritic cells.(88) Other antibody-based drugs used as immunosuppressants in transplantation include antithymocyte globulin (polyclonal), alemtuzumab (anti-CD52), and rituximab (anti-CD20).(reviewed in 89) A recent review by Cekic & Linden gives a detailed overview of purinergic signaling in specific immune cells following tissue injury (reviewed in 90).

Relevance in preclinical research and for clinical practice

Ectonucleotidases (CD39/CD73)

As described in the preceding sections, CD39 and CD73 are both abundantly expressed on T-reg cells,(91, 92) which allow them to hydrolyze the pro-inflammatory danger signal ATP to the anti-inflammatory mediator adenosine. Evidence for this protective role of CD39 was observed in a skin allograft rejection model, where the adoptive transfer of T-reg cells from CD39-deficient mice failed to prevent skin allograft rejection compared to the transfer of wild type T-reg cells.(92)

This protective effect of CD39 appears to involve adenosine production as activation of the adenosine 2A receptor has been shown to enhance survival of skin allografts in mice.(93) Several immune cells express CD73, such as subsets of B and T lymphocytes (94), T-reg cells (92), MSCs (95), but also intestinal epithelial cells (96) and endothelial cells of capillaries and venules (97). CD73 generates nucleosides for the purine salvage pathway; neighbour cells can take up the nucleosides via facilitated diffusion and use the molecules to recover nucleic acid bases and subsequently synthesize new nucleotides to meet critical metabolic needs of the cell. Also, CD73 generates adenosine, which activates the seven-transmembrane domain G-protein-coupled P1 purinergic (adenosine) receptors.

The P1A1 and P1A3 receptors bind to a Gi protein and cause a reduced intracellular concentration of cAMP by inhibiting the adenylyl cyclase, whereas the P1A2a and P1A2b receptors bind to a Gs protein and increase the intracellular concentration of cAMP by stimulating the adenylyl cyclase. Also, P1A2b and P1A3 receptors can interact with a Gq protein and stimulate the phospholipase C (reviewed in 98). However, signaling cascades of adenosine receptors are much more complex since they have also been shown to modulate protein kinase C, phospho-inositide 3 kinase and mitogen activated protein (MAP) kinases (reviewed in 99). P1 receptors have individual affinities towards their substrate. While the half maximal effective concentration (EC50) of the P1A1, P1A2a and P1A3 receptor is 0.01-1.00 μM , activation of the P1A2b receptor requires adenosine levels $>10 \mu\text{M}$ (EC₅₀ 24 μM). This means that the P1A2b receptor is not activated under physiological conditions but plays a role rather only when adenosine concentration is increased due to cellular stress such as in the case of inflammation (98).

In the majority of clinically relevant models, adenosine provides an anti-inflammatory signal and counteracts the pro-inflammatory reactions induced by the presence of ATP in the extracellular space. CD73 and its product adenosine have also been implicated as regulatory mechanisms in rejection of cardiac and tracheal allografts. In a model of heterotopic murine cardiac transplantation, CD73-deficiency of either donor or recipient resulted in significantly reduced allograft survival.(39) These observations are consistent with results from earlier studies which showed involvement of adenosine in the suppression of pro-inflammatory cytokine production.(100, 101) 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 P1A2b receptor.

The role of CD73 and endogenous adenosine was investigated in a study of murine acute GvHD with a MHC major mismatch between donor and recipient.(64) A CD73 deficiency in either donor or recipient mice resulted in significantly more severe GvHD, with reduced survival of the recipient, increased GvHD histopathology scores, and increased concentrations of IL-6 and interferon- γ in the serum of recipient mice compared to wild type mice. Furthermore, deletion of CD73 resulted in increased proliferation of alloreactive CD4⁺ and CD8⁺ T cells. These data are consistent with previous reports showing that even low concentrations of extracellular adenosine and other P1 receptor agonists inhibit T-cell activation and expansion via binding to the P1A2a receptor.(102) Furthermore, endogenous adenosine binding to the P1A2a receptor limited the expansion of alloreactive T cells and dampened the severity of acute GvHD. These findings extend on those from previous studies,(103) which suggested that activation of the P1A2a receptor via the selective agonist ATL146e improved the survival of GvHD mice without affecting the donor cell engraftment. Furthermore, the treatment of T cells with ATL146e reduced *in vitro* migration towards the chemokines CCL20, CXCL-12, and CXCL10 by at least 30%, while *in vivo* administration of ATL146e decreased the serum levels of various pro-inflammatory cytokines. P1A2a receptor activation also improved the clinical condition of mice with already established GvHD by reversing weight loss.(103)

Organ and graft heterogeneity

Transplant rejection due to organ and graft heterogeneity is a major obstacle in clinical practice, and strategies to improve tolerance include the suppression of T-cell and B-cell effector functions in addition to the activation of immunosuppressive T-reg cells.(104) Lung, cardiac, renal, and liver allografts (and also xenografts) exhibit quite distinct patterns of vascular inflammation and thrombosis that may dictate differential outcomes following transplantation. In prior studies of pig-to-primate xenotransplantation, protective-type genes (heme oxygenase-I, superoxide dismutases and CD39), together with thromboregulatory genes such as von Willebrand factor and P-selectin, were noted to be substantially upregulated in renal grafts that were associated with platelet consumption.(105) In cardiac xenografts, fibrin deposition was quite marked, and associated with increased plasminogen activator inhibitor-1 expression. The different outcomes observed in clinical practice might reflect overall differences in gene expression profiles, including those of purinergic signaling components, in renal and cardiac grafts.(105, reviewed in 106)

Xenotransplantation

The use of xenotransplantation in clinical practice has the potential to overcome the shortfall of available human organs and tissues for patients that urgently require transplantation.(reviewed in 107, 108) In the US alone, 18 potential transplant recipients die every day due to a lack of critical organs.(reviewed in 109) The pig is often cited as being the most suitable source of xenografts.(reviewed in 110) Research into the genetic manipulation of pigs to reduce xenograft rejection has focused largely on removing the Gal antigen from the surface of xenotransplant cells.(reviewed in 109) Increasing the expression of human CD39 and CD73 is also considered a potential strategy to prevent xenograft rejection by increasing the hydrolysis of ATP to adenosine and promoting immunosuppression.(reviewed in 107, 111) Additionally, CD39 is known to exert anticoagulant/antithrombotic properties, which may further aid in preventing xenograft rejection.

The current model for the pathogenesis of acute GvHD proposes that the disease develops in three stages. The first phase in acute GvHD is triggered by the cytotoxic chemotherapy or irradiation therapy of the recipient. This treatment causes necrotic and apoptotic cell death, particularly in the intestinal tract, leading to intestinal barrier breakdown, with subsequent activation of a local immune response including macrophages and other antigen-presenting cells (APCs). As a consequence, pro-inflammatory cytokines like tumour necrosis factor (TNF)- α and IL-1 β are released and neutrophil granulocytes are recruited into the intestinal tract, a process that is driven by the translocation of pathogen-associated molecular patterns, such as bacterially-derived lipopolysaccharide. In addition to these infectious stimuli, tissue destruction after the preconditioning treatment leads to release of specific non-infectious danger-associated molecular patterns, such as ATP.(reviewed in 112) 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 GvHD, transplanted donor T cells interact with APCs, most importantly with dendritic cells.(113) Local pro-inflammatory cytokines produced in the first GvHD phase serve as further stimuli for activation, differentiation, and proliferation of the donor-derived T cells.(reviewed in 114) In the third GvHD phase, after initial expansion the differentiated effector cells, mostly T cells, but also natural killer cells, macrophages, and neutrophils, migrate to the target tissues of GvHD (e.g. skin, GIT and liver) where they cause tissue destruction. ATP was recently shown to be released from necrotic 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.(63) Results from *in vivo* and *in vitro* models have shown that CD73 and endogenous adenosine significantly reduce the severity of acute GvHD.(64, 102, 103)

Conclusions

Purinergic signaling is closely associated with the regulation and direction of inflammation and local immune responses. Therefore, targeting the purinergic signaling pathway by increasing ectonucleotidase activity and/or boosting short term adenosine-mediated immunosuppression holds great potential in preventing allograft vascular injury, thereby ameliorating rejection and promoting tolerance. Existing immunosuppressive agents such as CNIs, MPA, and mTOR inhibitors can interact with purinergic signaling pathways either directly or indirectly. These

interactions provide great opportunities for the design of possible combination therapies with purinergic receptor agonists/antagonists to further manipulate and optimize immunosuppression in transplantation.

Based on studies in transplantation models, CD39 and other components of purinergic signaling pathways may be considered potentially crucial regulators of vascular inflammation, thrombosis, and tolerance induction. Therefore, further studies to increase our understanding of purinergic signaling in transplantation are of vital importance to clinical practice.

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Declaration of interests

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Search strategy and selection criteria

Data for this Review were identified by searches of PubMed and MEDLINE, and references from relevant articles using the search terms “purinergic”, “transplantation”, and “immunosuppression”. Only full-length articles published in English were considered with no date restriction. Additional articles were included at the discretion of the authors based on their experience in this field.

Figure legends

Figure 1. Overview of purinergic signaling. Intracellular ATP is released from cells through the opening of pannexin hemi channels, or via P2X7 receptors, where it can serve as an extracellular signaling molecule. ATP subsequently signals with various P2 receptors, both ligand-gated P2X and G-protein-coupled P2Y receptors, in an autocrine manner, which has been implicated in a wide variety of physiological processes. Eventually, ATP is hydrolyzed by ENTPDases expressed on cell surfaces, including CD39 and the ecto-5'-nucleotidase CD73, which promote the generation of adenosine. Adenosine signals on G-protein-coupled P1 receptors and is metabolized to inosine by the enzyme adenosine deaminase.

Figure 2. Distribution of purinergic receptors. An overview of purinergic receptor, CD39, and CD73 expression is shown in selected organs, tissues and cells. Further information on the distribution of purinergic receptors can be found in Table 1.

Figure 3. Immunosuppressive drugs and purinergic signaling. The intracellular or extracellular site of action of immunosuppressive drugs is shown. Green arrows indicate a stimulatory effect. Red lines indicate an inhibitory effect.

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Table 1. Purinergic receptor subtype properties.				
Receptor	Main distribution	Agonists	Antagonists	Transduction mechanisms
P1				
P1A1	Brain, spinal cord, testis, heart, autonomic nerve terminals	CCPA>R-PIA=S-ENBA, CVT-510, GR79236, 2'-MeCCPA, SDZ WAG 994, INO-8875, MRS 5474	DPCPX, N-0840, MRS1754, WRC-0571, PSB36, SLV320, CGS 16943	G _i /G _o ↓cAMP
P1A2a	Brain, heart, lungs, spleen	HENECA>CGS 21680=CVT-3146, ATL-146e, Regadenoson	KF17837, SCH58261, ZM241385, KW 6002	G _s ↑cAMP
P1A2b	Large intestine, bladder	Bay60-6583, NECA	PSB603, MRE-2029-F20, MRS1754, PSB0788, MRS1706, PSB1115, Alloxazine, GS-6201	G _s ↑cAMP, G _q /G ₁₁
P1A3	Lung, liver, brain, testis,	IB-	MRS1220, L-268605,	G _i /G _o , G _q /G ₁₁ , ↓cAMP,

	heart	MECA>MRS5698>MRS5168> 2-Cl-IB-MECA, DBXRM, VT160, HEMADO	MRS1191, MRS1523(rat), VUF8504, VUF5574, MRS1334(human), PSB10	PLC- β activation
P2				
P2X1	Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons	BzATP > ATP = 2- MeSATP $\geq\alpha,\beta$ -meATP=L- β,γ - meATP (rapid desensitization), PAPET-ATP	NF864>NF449>IP ₅ I ₂ TNP- ATP> RO 0437626> NF279, NF023, RO1, MRS2159	Intrinsic cation channel (Ca ²⁺ and Na ⁺)
P2X2	Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia, pancreas	ATP $\geq$ ATP γ S $\geq$ 2- MeSATP>> α,β -meATP (pH + zinc sensitive), β,γ -CF ₂ ATP	PSB-1011>RB2, isoPPADS>PPADS>Suramin , NF770, NF778, Aminoglycoside	Intrinsic ion channel (particularly Ca ²⁺)
P2X3	Sensory neurones, NTS, some sympathetic neurons	2-MeSATP $\geq$ ATP $\geq$ Ap ₄ A $\geq$ α,β -meATP (rapid desensitization), PAPET-ATP, BzATP	TNP-ATP, AF353, A317491, RO3, RO4, isoPPADS > NF110 > PPADS, Ip ₅ I, phenol red, RN-1838, Spinorphin	Intrinsic cation channel
P2X4	CNS, testis, colon, endothelial cells, microglia	ATP>> α,β -meATP>> CTP, 2- MeSATP Ivermectin potentiation	5-BDBD>>TNP-ATP, PPADS>BBG, Paroxetine, phenolphthalein, CO donor (CORM 2), 5MPTP	Intrinsic ion channel (especially Ca ²⁺)
P2X5	Proliferating cells in skin, gut, bladder, thymus, spinal cord, heart, adrenal medulla	ATP=2- MeSATP=ATP γ S>> α,β - meATP>AP ₄ A	BBG>PPADS, Suramin	Intrinsic ion channel
P2X6	CNS, motor neurons in spinal cord	- (only functions as a heteromultimer)	-	Intrinsic ion channel
P2X7	Immune cells including dendritic cells (mast cells, macrophages), pancreas, skin, microglia	BzATP>ATP $\geq$ 2-MeSATP >> α,β -meATP (clemastine potentiates)	KN62, BBG, KN04, MRS2427, O-ATP, RN- 6189, Perazine, AZ10606120, A740003, A- 438079, A-804598, GSK- 1370319, Comp 31 (GSK), AZD-9056, CE-224,535, JNJ-47965567, JNJ- 42253432 (penetrates BBB), decavanadate	Intrinsic cation channel and a large pore with prolonged activation
P2Y1	Epithelial and endothelial cells, platelets, immune cells, osteoclasts, brain	MRS2365>2- MeSADP=Ap ₅ (γ B)>>ADP β S> ATP > 2-MeSATP=ADP	MRS2500 > MRS2279 > MRS2179, PIT, A3P5P	G _q /G ₁₁ , PLC- β activation
P2Y2	Immune cells, epithelial and endothelial cells, kidney tubules, osteoblasts	2-thio-UTP>UTP, MRS2698 $\geq$ ATP, INS 365> INS 37217, UTP γ S>Ap ₄ A>MRS	AR-C126313>Suramin> RB2, PSB-716, MRS2576, PSB-0402	G _q /G ₁₁ and possibly G _i /G _o , PLC- β activation

		2768, Up ₄ -phenyl ester		
P2Y4	Endothelial cells, placenta, spleen, thymus	2'-azido-dUTP> UTP _γ S, UTP≥ATP≥ Ap ₄ A Up ₄ U MRS4062	ATP (human)>Reactive Blue 2>Suramin, MRS2577, PPADS	G _q /G ₁₁ and possibly G _i , PLC-β activation
P2Y6	Airway and intestinal epithelial cells, placenta, T cells, thymus, microglia (activated)	MRS2693> UDPβS, PSB0474> INS48823, Up ₃ U, 3-phenacyl-UDP>>UDP>UTP>>ATP, α,β-meUDP, MRS2957, MRS4129, 5-OMe-UDP αB	MRS2578>Reactive Blue 2, PPADS, MRS2567, MRS2575 (human)	G _q /G ₁₁ , PLC-β activation
P2Y11	Spleen, intestine, granulocytes	ATP _γ S>AR-C67085MX>BzATP≥ATP, NF546, NAD ⁺ , NAADP ⁺ , Sp-2-propylthio-ATP-α-B	NF157>Suramin>RB2, 5'-AMPS, NF340, AMP-α-5,	G _q /G ₁₁ and G _s , PLC-β activation
P2Y12	Platelets, glial cells	2-MeSADP≥ADP>ATP, ADP-β-S	AR-C69931MX> AZD6140 (Ticagrelor), INS50589>RB2>2-MeSAMP AR-C66096, CT50547, PSB-0413, Carba-nucleosides, MRS2395, AR-C67085, [³ H]PSB-0413, clopidogrel, AZD1283	Gα _i , inhibition of adenylate cyclase
P2Y13	Spleen, brain, lymph nodes, bone marrow, erythrocytes	ADP=2-MeSADP>2-MeSATP, ATP	AR-C69931MX>AR-C67085>MRS2211, 2-MeSAMP	G _i /G _o
P2Y14	Placenta, adipose tissue, stomach, intestine, discrete brain regions, mast cells	MRS2690>UDP>UDP glucose≥UDP-galactose, UDP-glucosamine, MRS2905	PPTN PZB01415033	G _q /G ₁₁
GPR17	Oligodendrocytes	Uracil nucleotides/cysteinyl-leukotrienes, MDL29,951	PZB01415033	G _i , adenylate cyclase inhibition
<p>Abbreviations: Ap5(yB), Adenosine pentaphosphate γ-boranophosphate; BBG, Brilliant blue green; 5-BDBD, 5-(3-Bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-one BzATP, 2'-&3'-O-(4-benzoyl-benzoyl)-ATP; cAMP, cyclic AMP; CCPA, chlorocyclopentyl adenosine; CTP, cytosine triphosphate; IP3, inosine triphosphate; Ip5I, di-inosine pentaphosphate; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, 5'-N-ethylcarboxamido adenosine; PLC, phospholipase C; RB2, reactive blue 2; P2X receptor subtype agonist potencies based on rat preparations, while P1 and P2Y receptor subtype agonist potencies are based on human preparations. Table is modified with permission from Burnstock 2003. (115)</p>

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