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The Immunosuppressive Role of Adenosine A_{2A} Receptors in Ischemia Reperfusion Injury and Islet Transplantation

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Abstract

Activation of adenosine A_{2A} receptors (A_{2A}R) reduces inflammation by generally inhibiting the activation of pro-inflammatory cells, decreasing endothelial adhesion molecule expression and reducing the release of proinflammatory cytokine mediators. Numerous preclinical studies using selective A_{2A}R agonists, antagonists, A_{2A}R knockout as well as chimeric mice have suggested the therapeutic potential of A_{2A}R agonists for the treatment of ischemia reperfusion injury (IRI) and autoimmune diseases. This review summarizes the immunosuppressive actions of A_{2A}R agonists in murine IRI models of liver, kidney, heart, lung and CNS, and gives details on the cellular effects of A_{2A}R activation in neutrophils, macrophages, dendritic cells, natural killer cells, NKT cells, T effector cells and CD4⁺CD25⁺FoxP3⁺ T regulatory cells. This is discussed in the context of cytokine mediators involved in inflammatory cascades. Whilst the role of adenosine receptor agonists in various models of autoimmune disease has been well-documented, very little information is available regarding the role of A_{2A}R activation in type 1 diabetes mellitus (T1DM). An overview of the pathogenesis of T1DM as well as early islet graft rejection in the immediate peri-transplantation period offers insight regarding the use of A_{2A}R agonists as a beneficial intervention in clinical islet transplantation, promoting islet graft survival, minimizing early islet loss and reducing the number of islets required for successful transplantation, thereby increasing the availability of this procedure to a greater number of recipients. In summary, the use of A_{2A}R agonists as a clinical intervention in IRI and as an adjunct to clinical immunosuppressive regimen in islet transplantation is highlighted.

Keywords

Adenosine A_{2A} Receptors; Cytokines; Inflammation; Ischemia reperfusion injury; Islet transplantation; Immune-suppression; Regulatory T cells; Islet graft survival; Type 1 diabetes

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CONFLICT OF INTEREST

The authors(s) confirm that this article content has no conflict of interest.

1. INTRODUCTION

Adenosine is a potent autocrine and paracrine immunosuppressive nucleoside that is released in the vicinity of damaged cells in conditions of metabolic stress such as hypoxia, ischemia, tissue injury or inflammation [1-2]. It can also be generated by the extracellular metabolism of adenine nucleotides. In the extracellular space, adenosine is involved in regulating both the innate and adaptive immune response *via* four widely expressed G protein-coupled receptors designated: A₁, A_{2A}, A_{2B} and A₃ [3]. Adenosine receptors are found on virtually all immune cells including polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, dendritic cells (DCs), lymphocytes and platelets, as well as endothelial cells [2]. Using quantitative RT-PCR, the expression of all four receptor transcripts has been demonstrated in granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic precursor cells populations [4]. Adenosine receptor occupancy in the majority of experimental systems activates an endogenous immunosuppressive pathway that acts to reduce tissue injury and inflammation and promote repair *via* four general modes, namely, increasing oxygen supply/demand ratio, preconditioning/postconditioning [5], anti-inflammatory effects [5,6-7] and stimulation of angiogenesis [5,8]. *In vitro* proliferation assays and mixed lymphocyte cultures demonstrate the ability of adenosine to decrease lymphocyte activation [9]. Adenosine receptor activation on monocytes, macrophages, and DCs has been documented to decrease the secretion of many of proinflammatory mediators including tumor necrosis factor- α (TNF- α), chemokine (C-C motif) ligand 3 and 4 (CCL3 and CCL4), interleukin (IL)-12, and nitric oxide (NO) [10]. While both A_{2A} and/or A_{2B} receptors have been implicated in the suppressive effects of adenosine on lymphocyte proliferation as well as cytokine production [5,7,11], the actual receptor sub-type involved in transducing the anti-inflammatory signal is determined largely by the cell type, organism and model being studied [3,12].

Gs-coupled Adenosine A_{2A} Receptors

A_{2A}Rs are found on most bone marrow-derived cells including, but not limited to, macrophages, monocytes, DCs, mast cells, eosinophils, T lymphocytes (CD4⁺ and CD8⁺ T cells), platelets, natural killer (NK) cells, natural killer T (NKT) cells and PMNLs [13-16]. Numerous studies using selective A_{2A}R agonists, antagonists as well as A_{2A}R knockout (A_{2A}R^{-/-}) animals, have highlighted the antiinflammatory/immunosuppressive role of A_{2A}R activation in various diseases [1,6-7,14-21]. These range from IRI, sepsis, and immune/inflammation-induced organ injury in diseases such as asthma, chronic obstructive pulmonary disease (COPD), myocardial infarction, Crohn's disease, rheumatoid arthritis, multiple sclerosis, colitis and hepatitis. Interestingly, the function of adenosine receptors in regulating autoimmune diabetes remains largely unexplored. This overview offers a mechanistic appraisal of the beneficial role of A_{2A}R activation as a therapeutic intervention in IRI as well as in clinical islet transplantation. We summarize evidence indicating that A_{2A}R activation enhances islet graft survival and function post-transplantation.

II. Pathogenesis of Ischemia Reperfusion Injury

Reperfusion injury refers to the damage that occurs in tissues upon restoration of blood flow following a period of ischemia. Reperfusion is characterized by the generation of reactive oxygen species (ROS), release of cytokines, induction of adhesion molecules on vascular endothelial cells, and the adhesion and extravasation of leukocytes into postischemic tissue [22]. These inflammatory events disrupt the integrity of the vascular endothelium and sinusoids and promote platelet aggregation, immunocyte activation, chemokine/cytokine induction and secretion and complement activation [23-24]. Several chemokines that are induced by IRI act as activators of neutrophil and monocyte diapedesis in the early stages of reperfusion injury [25], possibly contributing to IRI-induced inflammation. Tissue damage initiated during the ischemic period progresses during the reperfusion period.

Anti-inflammatory Responses of A_{2A}R in Ischemic Reperfusion Injury

Treatment with A_{2A}R agonists has been shown to be successful in abrogating 30-75% of the tissue injury associated with IRI [5,13] in liver [26], kidney [24-28], lung [29], heart [30], skin [31] and the spinal cord [32] by reducing neutrophil accumulation, preventing the release of pro-inflammatory cytokines and oxygen radicals, preventing endothelial cell activation, and greatly reducing microvascular occlusion, which can exacerbate tissue injury during reperfusion of previously ischemic tissues [14,26,33]. These cellular responses seem to be mediated predominantly by cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)-dependent pathways [16,34-35].

Liver

Early studies demonstrated that the mechanism of hepatic IRI involved CD4⁺ T cell activation with secretion of cytokines such as TNF- α , interferon- γ and IL-6 [12,26,36] that resulted in liver cell damage, apoptosis, necrosis and subsequently liver death. ATL146e, a potent A_{2A}R agonist, was shown to inhibit liver damage and concanavalin A (ConA)-induced elevation in serum cytokine levels [23,26] while A_{2A}R^{-/-} mice displayed enhanced ConA-induced liver inflammation and necrosis [23,37]. A number of studies have confirmed that ATL146e and other A_{2A}R agonists reduced inflammation and afforded tissue protection in hepatic IRI by inhibiting chemokine and cytokine production [19,22-23,26,38]. For instance, administration of ATL146e in wild-type (WT) but not in A_{2A}R^{-/-} mice suppressed the production of IL-1 α , IL-1 β , IL-1R α , IL-6, IL-10, IL-18, IFN- β , IFN- γ as well as CC and CXC chemokines CCL2, CCL3, CCL4, CCL5, chemokine (C-X-C motif) ligand 2 (CXCL2) and IFN- γ -inducible protein-10 (IP-10), probably contributing to tissue protection, since protection afforded by ATL146e administered at the time of reperfusion was blocked by the selective A_{2A}R antagonist ZM241385 [19,23]. Interestingly, CCL2, CCL5 and IP-10 are all inducible chemokines that are chemotactic to T lymphocytes. Similar studies have demonstrated that activation of the A_{2A}R inhibits the secretion of pro-inflammatory cytokines, IL-12 and TNF- α [39]. In a rat model of small-for-size liver transplantation, A_{2A}R expression increased after transplantation, suppressing NF- κ B activation and decreasing the expression of TNF- α , CXCL2 and cluster of differentiation 54 [CD54, also known as intercellular adhesion molecule-1 (ICAM-1)] [40].

Whilst these studies demonstrated that protection by A_{2A}R agonists was mostly attributable to activation of A_{2A}Rs on CD4⁺ T cells and to the inhibition of T cell receptor (TCR)-mediated IFN- γ production, the rapidity of the IRI led Lappas *et al.* to speculate that it was activation of the invariant natural killer T (iNKT) cell subset, by a CD1d-restricted mechanism, that probably played a central role in initiating the inflammatory cascade and was the target of A_{2A}R agonists [22]. iNKT cells recognize lipid antigens derived from pathogens or possibly damaged tissue that are presented by the major histocompatibility complex (MHC) class I-related molecule CD1d, by antigen presenting cells (APCs). Lappas *et al.* demonstrated that within two hours after the initiation of reperfusion, NKT cells were stimulated to produce IFN- γ . The use of antibodies to deplete NK1.1⁺ cells (NK and NKT) or to block CD1d-restricted antigen presentation to NKT cells replicated, but was not additive to, the protection afforded by ATL146e. The adoptive transfer of NKT cells purified from WT or A_{2A}R^{-/-} mice but not IFN- γ ^{-/-} mice, restored reperfusion injury to the WT level in RAG-1^{-/-} mice. Recipient animals with transferred A_{2A}R^{-/-} NKT cells were not protected from hepatic reperfusion injury by ATL146e. Also, ATL146e was capable of potentially inhibiting IFN- γ production in NKT cells in response to anti-CD3 antibodies or the CD1d-restricted glycolipid antigen, α -galactosylceramide (a-GalCer). Thus, the authors concluded that activation of iNKT cells initiated the recruitment and transactivation of other immune cells such as macrophages and neutrophils that propagated tissue inflammation and injury. It is now known that all four types of adenosine receptors A_{1A}R, A_{2A}R, A_{2B}R, and A₃R are present on mouse iNKT cells and while adenosine can suppress IFN-g production by iNKT cells, A_{2A}R activation is required for the secretion of IL-4 and IL-10 by these cells [41]. This is supported by the observation that A_{2A}R^{-/-} mice exhibited largely decreased levels of IL-4, IL-10 and transforming growth factor-b (TGF-b) concomitantly with an increase of IFN-g upon a-GalCer administration *in vivo* [41]. Thus, while A_{2A}R activation inhibits other lymphocyte populations, it is required for the secretion of IL-4 and IL-10 by iNKT cells. Interestingly, Chouker *et al.* have demonstrated that total body hypoxia-triggered pathway provided protection in acute hepatitis and that hypoxia (upstream) and A_{2A}R (downstream) functioned in the same immunosuppressive and liver tissue-protecting pathway [37].

Kidney

A_{2A}Rs have been found in the glomeruli of the kidney, in the mesangial and the tubular epithelial cells as well as in the kidney microvasculature [42]. Improved renal morphology and function following ATL146e administration in renal IRI has been observed in mice and rats, reducing injury by 70–80%. Day *et al.* have demonstrated that the protective effect of ATL146e was abrogated in chimeric mice in which bone-marrow of WT mice was ablated and reconstituted with bone marrow from A_{2A}R^{-/-} animals (A_{2A}^{-/-}R⁻→WT), but was intact in WT→WT chimeras [43]. These results indicated that the protective effect of A_{2A}R agonists was mediated by A_{2A}Rs expressed on bone-marrow derived cells. ATL146e reduced the induction of IL-6, IL-1b, IL-1 receptor antagonist (IL-1ra), and transforming growth factor-a (TGF- α) mRNA in WT→WT chimeras, but not in A_{2A}R^{-/-}→WT chimeras. Kidneys from macrophage-depleted mice reconstituted with macrophages lacking A_{2A}Rs were still protected by ATL146e, ruling out involvement of macrophage A_{2A}R activation in reducing IRI [42,44]. Interestingly, macrophages do appear to play a role in the protective

effect of A_{2A}R activation in diabetic nephropathy [45]. The activation of A_{2A}Rs expressed on macrophages in nephritic glomeruli with CGS21680 (also a potent A_{2A}R agonist) prevented progressive kidney fibrosis in a rat model of immune-associated chronic nephrotoxic nephritis by reducing macrophage infiltration and suppressing the expression of the macrophage-linked glomerular damage mediators, TGF- β , osteopontin-1, thrombospondin-1, and tissue inhibitor of metalloproteinase-1 [46]. CGS21680 also prevented the progression of lupus nephritis in MRL/lpr mice by significantly attenuating renal macrophage and T cell infiltration, inhibiting NF κ B activation and suppressing the expression of IFN- γ , CCL2 and MHC-class II in MRL/lpr splenocytes [47]. Day *et al.* demonstrated that the mechanism of protection of ATL146e in IRI was *via* stimulation of A_{2A}Rs on CD4⁺ T cells and inhibition of IFN- γ release, similar to that observed in hepatic IRI [27]. Rag-1^{-/-} mice subjected to ischemia-reperfusion were protected in comparison to WT mice. Furthermore, ATL146e did not confer additional protection. However, adoptive transfer of WT CD4⁺ T cells or A_{2A}-/-R CD4⁺ T cells reversed the protection observed in the Rag-1^{-/-} mice. ATL146e reduced injury in Rag-1^{-/-} mice that had been adoptively transferred with WT CD4⁺ T cells but not in those that received A_{2A}R^{-/-} CD4⁺ T cells. Additionally, Rag-1^{-/-} mice reconstituted with IFN- γ ^{-/-} CD4⁺ T cells were also protected from IRI, whilst ATL146e conferred no additional protection. An interesting observation was that marked neutrophil infiltration of the outer medulla was observed following IRI of Rag1^{-/-} mice adoptively transferred with WT CD4⁺ T cells or A_{2A}R^{-/-} CD4⁺ T cells. However the ability of A_{2A}R agonists to block neutrophil infiltration depended on the presence of A_{2A}R expressed on CD4⁺ T cells. When reconstitution of Rag-1^{-/-} mice was conducted with CD4⁺ T cells from IFN- γ ^{-/-} mice, there was marked reduction of neutrophil infiltration. These results confirm the relationship between T cells and neutrophils and suggest the participation of neutrophils in the early phase (<24 h) of ischemia-reperfusion [48]. Other studies have correlated decreased endothelial cell adhesion molecule expression, neutrophil sequestration in kidney parenchyma as well as improved circulation due to vasodilation of renal microvasculature with A_{2A}R-mediated renal protection [49]. Taken together, CD4⁺ T lymphocytes play an important role in A_{2A}R-mediated tissue protection and a complex interplay between CD4⁺ T lymphocytes and other lineages such as macrophage/monocyte, platelet, and endothelium in recruitment of neutrophils exists at the first few hours of reperfusion [50].

Heart

The inflammatory response induced during myocardial IRI causes a dramatic increase in neutrophil adherence to the reperfused endothelium leading to progressive capillary plugging, thereby preventing full restoration of capillary flow upon reperfusion. Several studies have reported the cardioprotective effect of A_{2A}R activation in reducing infarct size and downregulating proinflammatory responses in myocardial IRI [51-52]. Using ATL-146e in WT, A_{2A}R^{-/-}, Rag-1^{-/-} and chimeric mice specifically lacking A_{2A}R on bone marrow-derived cells, it was observed that while reduction of infarct size was associated with corresponding inhibition of P-selectin induction, neutrophil infiltration and other indices of inflammation [51], the cardioprotective effect of A_{2A}R activation was mediated primarily through inhibition of T lymphocytes rather than directly through the inhibition of neutrophils or monocytes [30,53]. Activation of A_{2A}R on CD4⁺ T lymphocytes not only

inhibited rapid accumulation of T lymphocytes that occurred in ischemic region of the myocardium within 2 minutes of reperfusion, but also the extent of reperfusion injury, neutrophil accumulation, and IFN- γ released. Interestingly, while an increase in infarct size to WT (C57BL/6 mice) level was observed in Rag1^{-/-} mice by the adoptive transfer of CD4⁺ T lymphocytes derived from WT or A_{2A}R^{-/-} mice, ATL146e was only capable of blocking this effect in mice reconstituted with WT-derived CD4⁺ T cells and not with A_{2A}R^{-/-} CD4⁺ T cells. Also, adoptive transfer of CD4⁺ T lymphocytes derived from IFN- γ ^{-/-} mice was unable to increase infarct size in Rag1^{-/-} mice, which lack mature lymphocytes. These results indicated that A_{2A}R stimulation on CD4⁺ T cells not only attenuated neutrophil and T lymphocyte infiltration into the infarcted area but also decreased IFN- γ production by transmigrating T cells [30,53]. In *ex vivo*, buffer-perfused hearts, mast cell degranulation contributes to myocardial IRI [54]. A_{2A}R activation by ATL146e or CGS21680 contributed to cardioprotective effects in the isolated heart, as measured by decreased infarct size at least in part by attenuating resident mast cell degranulation. Studies investigating the role of A_{2A}Rs in aortic vascular tone indicated that A_{2A}R^{-/-} mice had significantly lowered aortic relaxation and endothelial function, indicating the important role of A_{2A}Rs in vasorelaxation, in part through an endothelium-dependent mechanism [55]. Regadenoson (Lexiscan) has been approved by the FDA as the first A_{2A}R agonist to be used clinically for vasodilator stress testing in conjunction with myocardial perfusion imaging [56]. Apadenoson, (Stedivaze, ATL146e) is in phase 3 development for the same indication [57]. Some studies suggest that A_{2B}Rs may also play an important role in modulating myocardial reperfusion injury and that a simultaneous stimulation of A_{2A}R and A_{2B}R with CGS21680 and adenosine A1/A2 receptor agonist NECA at reperfusion is required to produce a strong cardioprotection against reperfusion injury [58].

Lungs

Activation of A_{2A}Rs has been postulated as a possible treatment for lung inflammatory diseases such as asthma and COPD. In rats and mice, intra-tracheal or intra-nasal administration of CGS21680 has been shown to suppress lung inflammation induced by sensitization to ovalbumin in a allergic asthma model [16,59-60]. The immunosuppressive process involved inhibition of neutrophil, eosinophil, macrophage and lymphocyte infiltration. Fozard *et al.* demonstrated that CGS21680 manifested a broad-spectrum, dose-dependent, anti-inflammatory activity through activation of A_{2A}Rs [59]. This was manifested as suppression of leukocyte influx into broncho-alveolar lavage (BAL) fluid and a decrease in the myeloperoxidase and eosinophil peroxidase activities and protein concentrations. Intratracheally-administered CGS21680 also reduced blood pressure in anaesthetised rats at doses similar to those at which anti-inflammatory effects were manifested. Both the anti-inflammatory and hypotensive responses were blocked by pretreatment with ZM241385, a selective adenosine A_{2A}R antagonist, confirming their mediation by the A_{2A}R subtype. Bonneau *et al.* also observed similar results in a mouse model of asthma, wherein CGS21680 given therapeutically after repeated ovalbumin challenge inhibited inflammatory cell influx into the BAL fluid but had no effect on the allergen-induced bronchoconstriction, airway hyperreactivity or the BAL fluid mucin levels [60]. In a lipopolysaccharide (LPS)-driven model however, CGS21680 had no effect on the BAL fluid inflammatory cell influx or TNF- α , keratinocyte chemoattractant and CXCL2

levels, but potently inhibited neutrophil activation [61]. A recent study using an isolated, buffer-perfused murine lung model indicated that treatment with A_{2A}R agonists attenuated the significant induction of TNF- α , CXCL1, CXCL2 and CCL5 production following ischemia reperfusion [62]. In contrast, lungs of A_{2A}R^{-/-} mice were not protected by the A_{2A}R agonist, ATL313. In an *in vivo* model of lung IRI, A_{2A}R activation on CD4⁺ T lymphocytes and neutrophils attenuated lung IRI and reduced the expression of TNF- α , IL-17, KC, CCL2, CCL3, CCL4 and CCL5 [63-64]. Together, these results suggest that A_{2A}R activation exerts its protective effect by inhibiting inflammatory cell influx and reducing inflammatory cell activation in asthma and COPD respectively.

A major iatrogenic complication of oxygen therapy in conditions of acute lung inflammation is the oxygenation-associated elimination of local tissue hypoxia-driven and A_{2A}R-mediated anti-inflammatory tissue protective mechanisms. Using A_{2A}R^{-/-} mice and A_{2A}R antagonist-treated WT mice, Thiel *et al.* demonstrated the critical role of A_{2A}R activation in providing lung protection from enhanced neutrophil accumulation, lung vascular permeability and impairment of lung gas exchange [61]. They observed similar lung-protective effects brought about by hypoxia and elegantly demonstrated that hypoxia occurred in upstream of A_{2A}Rs in the same anti-inflammatory, lung tissue-protecting pathway, as shown by the failure of the adenosine-producing hypoxia to protect A_{2A}R-deficient mice from death and excessive pulmonary TNF- α cytokine production. Furthermore, they provided confirmation of the hypoxia-elicited lung-protective effects by rescuing mice from hyperoxia-accelerated death with CGS21680. In a study designed to examine the effect of A_{2A}R signaling on tracheal rejection in a mouse model of *Bronchiolitis obliterans*, A_{2A}R agonist protected against lymphocyte infiltration and luminal occlusion [64]. Nitrosative stress produced by inflammatory cells is also associated with the pathophysiology of asthma. Nadeem *et al.* observed that A_{2A}R deficiency led to enhanced airway inflammation and airway hyperresponsiveness, possibly *via* involvement of nitrosative stress in a mouse model of asthma [65]. The use of UK371,104 an A_{2A}R receptor agonist that remains in the lung after pulmonary delivery may be useful for assessing the anti-inflammatory potential of inhaled A_{2A}R agonists for treatment of asthma [66]. A recent study indicates that pretreatment with A_{2A}R agonist ATL1223 attenuated reperfusion injury in a preclinical porcine lung transplantation model by reducing inflammation [67]. Significantly lower lung wet/dry weight, proinflammatory cytokine expression and lung injury scores by histology were observed with treatment, supporting the development of pharmacologic A_{2A}R agonists for use in human clinical trials for lung transplantation. Also, CGS21680 administration was shown to decrease acute lung inflammation in a mouse model of carrageenan-induced pleurisy, characterized by neutrophil infiltration of lung tissues, increased production of NO, TNF- α and IL-1 β and increased expression of CD54 and CD62P (platelet-adhesion molecule, P-selectin) [68]. Furthermore, carrageenan induced the expression of nuclear factor- κ B (NF- κ B), inducible nitric oxide synthase (iNOS) and nitrotyrosine as well as the activation of poly-ADP-ribosyl polymerase (PARP) and apoptosis [Fas-ligand (Fas-L) expression, Bcl-2 and Bcl-2 associated X protein (Bax) expression] in the lung tissues. CGS21680 not only caused a significant reduction in all parameters of inflammation, but also reduced neutrophil infiltration and the degree of lung injury. A_{2A}R agonists have also shown promise for treating diseases associated with iNKT or NK cell activation. Activation

of induced A_{2A}R on iNKT and NK cells in NY1DD mice with sickle cell disease mice was sufficient to improve baseline pulmonary function and prevent hypoxia-reoxygenation-induced exacerbation of pulmonary injury [69].

CNS

Li *et al.* showed that treating mice with ATL146e beginning before or just after the onset of reperfusion after compression-induced spinal cord injury rapidly and persistently reduced locomotor dysfunction and spinal cord demyelination [70]. Protection was abolished in A_{2A}R^{-/-}→WT bone marrow chimera mice selectively lacking the A_{2A}R only on bone marrow-derived cells but retaining receptors on other tissues including blood vessels. Other studies confirm that A_{2A}R activation acutely reduces reperfusion injury in spinal cord IRI [71]. In an experimental model of spinal cord trauma, A_{2A}R agonists ATL313 and CGS21680 administered after spinal cord injury reduced motor deficit as well as reduced tissue damage, TUNEL/Annexin-V staining, TNF-α expression and expression of apoptosis-related Bax, Fas-L and Caspase-3 and Caspase-3 genes [72]. These results demonstrated that A_{2A}R agonist therapy prevented the apoptotic process that is an important step of secondary damage after spinal cord injury. In direct contrast, A_{2A}R deficiency was shown to attenuate brain injury induced by transient focal ischemia in adult A_{2A}R^{-/-} mice compared to WT mice [73]. However, it is interesting to note that hypoxic ischemia resulted in aggravated brain damage in immature seven-day-old A_{2A}R^{-/-} mice suggesting A_{2A}Rs played a protective role against hypoxic ischemic brain injury in neonates [74]. It appears that A_{2A}R inactivation in most studies and activation in some, reduced brain injury and attenuated inflammation through modulation of proinflammatory cytokine production [75]. Similarly, opposing functions of A_{2A}Rs in striatal and extrastriatal neurons appear to fine-tune psychomotor activity [76]. A_{2A}R agonists have been proposed as potential therapeutic agents for schizophrenia [77] and other psychotic disorders [78].

A_{2A}R Signaling

Gs-protein-coupled A_{2A}Rs signal mainly by the adenylate cyclase–cAMP–PKA canonical pathway, but can also signal through the activation of an exchange factor that is directly activated by cAMP (Epac; Exchange protein activated by cAMP) [79]. Signaling downstream from PKA is in part by phosphorylated cAMP response element-binding (CREB) protein, which in turn mediates gene expression directly by interacting with gene promoters or indirectly by competing with NF-κB or other transcription factors for an important cofactor, CBP6 [80]. Phospho-CREB had been shown to inhibit NF-κB transcriptional activity and suppress proinflammatory cytokine expression (e.g., TNF) in immune cells [20]. Activation of mitogen-activated protein (MAP) kinases, protein kinase C (PKC) and protein phosphatases are other pathways of signaling by A_{2A}Rs [80-83]. For instance, signal transduction by adenosine in PMNs proceeds in part *via* cAMP-independent activation of a serine/threonine protein phosphatase in the plasma membrane resulting in the modulation of superoxide anion generation [82]. The PKC pathway has been implicated as a crucial mechanism that is required for the induction of hypoxia-inducible factor 1 in macrophages [83]. Other studies indicate that CCAAT-enhancer-binding proteins (C/EBPβ) are responsible for the stimulatory effect of A_{2A}R agonists on IL-10 production by

macrophages suggesting that the A_{2A}RC/EBPb axis is critical for IL-10 production after bacterial infection [84].

III. ANTI-INFLAMMATORY ROLE OF A_{2A}R ACTIVATION

Macrophages

Several studies have demonstrated that following macrophage activation, adenosine inhibits IL-12 and TNF- α production *via* adenosine A_{2A}R-dependent and independent mechanisms [1,85]. A_{2A}R activation was also associated with upregulation of LPS and *Staphylococcus* induced IL-10 secretion by human monocytes as well as mouse peritoneal macrophages [85-86]. Interestingly, both hypoxia and A_{2A}R stimulation act as potent inducers of vascular endothelial growth factor (VEGF) production by murine peritoneal macrophages [87]. However, synergistic interaction of A_{2A}R agonists through A_{2A}R with LPS through the toll-like receptor 4 (TLR4) pathway results in a strong up-regulation of VEGF expression by murine macrophages in a hypoxia- and NO-independent manner. Similar synergistic interaction between A_{2A}Rs and TLR2, TLR7 and TLR9 has also been observed [88].

Dendritic Cells

In immature DCs, A_{2A}Rs are expressed at low levels, and it is the activation of the A₁ and A₃ adenosine receptors that promotes recruitment of these cells to sites of inflammation and injury [89]. DC maturation is accompanied by the emergence of A_{2A}R-mediated signaling responses. Studies by Panther *et al.* indicate that activation of A_{2A}R on mature DCs resulted in enhanced CD54, CD80, MHC class I, and human leukocyte antigen HLA-DR molecule expression as well as a dose-dependent inhibition of TNF- α and IL-12 and augmentation of IL-10 secretion [90]. It also augmented the release of the chemokine CCL17 and inhibited CXCL10 production. DCs matured in the presence of adenosine had a reduced capacity to induce T helper cell type 1 (T_{H1}) polarization of naïve CD4⁺ T lymphocytes. Thus, activation of A_{2A}R causes a polarization of naïve CD4⁺ T cells towards a T_{H2} cell profile, with a shift towards an anti-inflammatory cytokine profile, with reduced IL-12, IL-6 and IFN- γ production and augmented IL-10 production [90].

Neutrophils

Numerous studies have demonstrated that activation of A_{2A}R inhibits recruitment of neutrophils to the site of inflammation by decreasing the expression of adhesion molecules expressed on neutrophils, thereby reducing firm adhesion of neutrophils to the endothelium [91]. A_{2A}R activation was shown to counteract stimulation of neutrophil CD49d integrin expression and neutrophil binding to CD106 [vascular cell adhesion molecule-1 (VCAM-1)] *via* a cAMP/PKA-mediated pathway [92]. At sites of inflammation, activated A_{2A}R diminished the formation of ROS, the secretion of a large range of cytokines/chemokines including TNF- α , CCL3, CCL4, CXCL2 and CCL20, of leukotriene B₄ and the expression of adhesion molecules [93-95], thereby decreasing tissue damaging activity of neutrophils [2]. A_{2A}R up-regulation in human neutrophils by T_{H1} cytokines TNF- α and IL-1 β possibly represents a potential feedback mechanism to control inflammation. It has recently been discovered that A_{2A}R expression in human PMNs is inversely correlated with the expression levels of miRNA-214, miRNA-15, and miRNA-16 [96].

Lymphocytes

A number of studies using $A_{2A}R^{-/-}$ mice as well as pharmacological agents indicate that $A_{2A}Rs$ play a dominant role in regulating lymphocyte responses and that the anti-inflammatory effects observed in animal models of autoimmunity and ischaemia are mediated, in part, by targeting lymphocytes. A very recent study has shown that $A_{2A}R$ activation of T effector cells by CGS21680 potently inhibited the release of IL-1, IL-2, IL-3, IL-4, IL-12, IL-13, IFN- γ , TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL3, and CCL4 but did not alter cytokine/chemokine release in $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T (T_{reg}) cells [97]. In a study that sought to examine the expression of adenosine receptors on human $CD4^{+}$ T cells from blood or gastric tissue and evaluate the role of $A_{2A}R$ in regulating gastritis following *helicobacter pylori* infection, it was observed that activation of $A_{2A}R$ inhibited pro-inflammatory cytokine production (TNF- α and IFN- γ) by human helper T cells and reduced *Helicobacter*-induced gastritis [98]. An interesting observation was that $A_{2A}R$ stimulation suppressed the development of T cell receptor (TCR)-stimulated naive T cells into both T_{H1} and T_{H2} cells [99]. Csoka *et al.* showed a decrease IFN- γ production in naïve T cells developed under T_{H1} -skewing conditions and decreased interleukin IL-4, IL-5, and IL-10 production by cells developed under T_{H2} -skewing conditions. Using $A_{2A}R^{-/-}$ mice, it was observed that $A_{2A}R$ activation inhibited T_{H1} - and T_{H2} -cell development by decreasing the proliferation and IL-2 production of naive T cells, irrespective of whether the cells are expanded under T_{H1} - or T_{H2} -skewing environment. These studies challenge the hypothesis that $A_{2A}Rs$ on lymphocytes might shift T_{H1} cell responses towards a T_{H2} profile. Seigny *et al.* demonstrated $A_{2A}R$ activation upregulated negative co-stimulatory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed cell death 1 (PD1) and down-regulated positive co-stimulatory molecule CD-40L [100]. These phenotypic changes induced by $A_{2A}R$ activation contributed to prolonged allograft skin survival.

Studies using A_1 -, A_{2A} - and A_3 -receptor $^{-/-}$ mice have also indicated a primary role for $A_{2A}Rs$ in preventing the cytolytic activity of IL-2-activated NK cells by interfering with both perforin-mediated and Fas-L-mediated killing pathways [101]. $A_{2A}Rs$ are also found on iNKT cells where they inhibit the activation of mouse $CD4^{+}NK1.1^{+}$ NKT cells that express an invariant V α 14J α 18 TCR resulting in substantial protection from injury caused by IFN- γ production as well as downstream events in the reperfusion-induced inflammatory cascade including neutrophil accumulation and tissue necrosis [22]. Activation of iNKT cells has been implicated in a number of disease processes including atherosclerosis, T1DM, arthritis and various allergic diseases [102]. The presence of $A_{2A}Rs$ on iNKT cells suggests that there could be novel clinical applications of $A_{2A}R$ agonists or antagonists in the treatment of these diseases.

Several studies indicate a role for adenosine in mediating the immunosuppressive properties of T_{reg} cells [103-104]. T_{reg} cells express high levels of cell surface ectoenzymes CD39 (Ectonucleoside triphosphate dephosphorylase 1) and CD73 (Ecto-5'-nucleotidase) that convert extracellular ATP and ADP to adenosine. T_{reg} cells also express $A_{2A}Rs$ and the coordinated expression of both CD39 and CD73 on T_{reg} cells and $A_{2A}Rs$ on activated T effector cells modulate the inhibitory function of T_{reg} cells [103]. This was supported by the

observation that T_{reg} cells from *Cd39*-null mice show impaired suppressive properties *in vitro* and failed to block allograft rejection *in vivo*. Naganuma *et al.* demonstrated that while adoptive transfer of T_{reg} cells from WT mice blocked colitis, T_{regs} obtained from A_{2A}R^{-/-} mice were unable to prevent disease [104]. Furthermore, A_{2A}R activation suppressed the expression of proinflammatory cytokines while sparing anti-inflammatory activity mediated by IL-10 and TGF- β . Another interesting observation is that T_{reg} cells isolated from CD39^{-/-} mice lose their potential to suppress proliferation of CD4⁺CD25 T cells. This property was restored upon reconstitution with soluble exogenous forms of CD39, highlighting the role of adenosine in mediating these effects. Furthermore, A_{2A}R^{-/-} CD4⁺CD25 T cells proliferated more when compared to WT cells when cocultured with T_{reg} cells, once again highlighting the suppressive effect of adenosine on proliferation of proinflammatory cells. A_{2A}R activation in T_{reg} cells has also been shown to upregulate FoxP3 expression [103]. Interestingly, Beldi *et al.* have shown that NKT cells, like T_{regs}, also express both CD39 and CD73 and can generate adenosine from extracellular nucleotides [105]. It is possible that the functional consequences of CD39 and CD73 coexpression on NKT cells might be comparable to that of T_{reg} cells and could account for the immunosuppressive properties of these cells. A recent study indicates that CD39 and CD73 on ovarian cancer cells are responsible for A_{2A}R dependent suppression of T cell function and NK cell cytotoxicity similar to the mechanism used by T_{reg} cells [106]. Ohta *et al.* also made an interesting observation that genetic deletion of A_{2A}R in the host resulted in rejection of established immunogenic tumors in $\approx 60\%$ of A_{2A}R-deficient mice with no rejection observed in control WT mice, indicating that targeting of the hypoxia $\rightarrow$ adenosine $\rightarrow$ A_{2A}R pathway may be a cancer immunotherapy strategy for preventing the inhibition of antitumor T cells in the tumor microenvironment [107]. However, the observation of autoimmunity during melanoma rejection in A_{2A}R-deficient mice also indicates that A_{2A}R activation in T cells may play an important role in preventing autoimmunity. Thus, this study demonstrates that while hypoxia $\rightarrow$ adenosine $\rightarrow$ A_{2A}R pathway inhibitors may improve antitumor immunity, the recruitment of this pathway by selective drugs may attenuate autoimmune tissue damage. Taken together, these studies indicate that A_{2A}R agonist-based therapies might be beneficial in the treatment of autoimmune and/or inflammatory diseases.

Despite an impressive collection of data highlighting the anti-inflammatory/ immunosuppressive role of A_{2A}R activation in various disease conditions, the function of A_{2A}Rs in modulating autoimmune T1DM remains largely unknown.

IV. PATHOGENESIS OF TYPE 1 DIABETES MELLITUS

T1DM is an organ-specific, multistage, T cell-mediated autoimmune disease that involves the slow and progressive destruction of pancreatic β -cells and a complete loss of insulin secretion [108]. Several studies indicate that innate immunity and non-specific inflammation might contribute to early induction and amplification of the immune assault against pancreatic β -cells and, at later stages, to the stabilization and maintenance of insulinitis [109-111]. During the course of the disease, inflammatory mediators probably contribute to the suppression of β -cell function and viability, may inhibit or stimulate β -cell regeneration, and cause peripheral insulin resistance. The involvement of β -cell autoantigens,

macrophages, DCs, T and B lymphocytes in the pathogenesis of this disease has been extensively detailed [108-111]. Recent studies have identified abnormalities in some of the innate effector cell population (such as monocytes/macrophages, DCs, NK cells, NKT cells and gdT cells) both in patients with T1DM and in those at risk of the disease, but whether these abnormalities predispose to the development of autoimmune diabetes is, as yet, uncertain [111]. APCs such as macrophages and/or DCs are the first cell types to infiltrate the pancreatic islets followed by CD4⁺ and CD8⁺ T cells and B cells. B lymphocytes play a critical role as APCs while macrophages have also been shown to play an essential role in the development and activation of cytotoxic CD8⁺ T cells. b-cell autoantigens released from cells during spontaneous turnover are processed by macrophages and presented to helper T cells in conjunction with MHC class II molecules. Activated macrophages secrete IL-12 which may activate T_{H1} type CD4⁺ T cells resulting in secretion of cytokines IFN- γ , TNF- α , TNF- β and IL-2, which in turn maximally activate CD8⁺ T cells to differentiate into CD8⁺ effector T cells [111-114]. Activated CD4⁺ and CD8⁺ T cells are the final effectors that activate b-cell death *via* apoptosis. Simultaneously, IFN- γ released by CD4⁺ and CD8⁺ T cells may in turn cause macrophages to release substantial amounts of cell-toxic cytokines and free radicals. These proinflammatory cytokines may induce the expression/upregulation of Fas on pancreatic b-cells [112-113] resulting in the induction of β -cell apoptosis by FasL-expressing CD4⁺ T cells, CD8⁺ T cells as well as secretion of pro-inflammatory cytokines [110]. The latter induce b-cell apoptosis through a canonical mitochondrial pathway, and this effect is dependent on calcineurin-mediated Bcl-2-associated death (Bad) promoter protein dephosphorylation and Bax protein activity [115]. Pro-inflammatory cytokines also induce calcineurin-dependent dephosphorylation of Bad Ser136, mitochondrial stress, cytochrome c release, activation of caspase-9 and -3, and DNA fragmentation. Inhibition of Bad Ser136 dephosphorylation or Bax was found to inhibit cytokine-induced intrinsic pro-apoptotic signaling. Ultimately, CD8⁺ T cell-mediated b-cell destruction is due to the interplay between receptor mediated (Fas-Fas-L, TNFTNF receptor) interactions, secretion of ROS and cytokines and the release of granzymes and perforin released from cytotoxic effector T cells, resulting in the development of auto-immune T1DM. Furthermore, DNA damage in b-cells leads to the activation of poly (ADP-ribose) polymerase which rapidly depletes NAD which is protective against radical induced necrosis [112]. Interestingly, transgenic mice with -cell specific overexpression of copper, zinc superoxide dismutase, or thioredoxin are resistant to autoimmune and streptozotocin-induced diabetes.

T1DM is thought to be a T_{H1}-dominance disease and it has been shown that type 1 cytokines such as INF- γ , TNF- α , IL-2, IL-12 and IL-18 derived from the T subset of CD4⁺ T cells are actively involved in β -cell destruction [116]. On the other hand, cytokines secreted by the T_{H2} and T_{H3} cells such as IL-4, IL-5, IL-10 and TGF- are thought to inhibit β -cell destruction. Thus, it appears that cytokines probably modulate the immunological response towards cytotoxic effector cell or suppressor cell dominance. Compelling evidence for a third effector CD4⁺ T_H pathway in autoimmunity, designated T_{H17} T cells that are generated by a combination of TGF- β 1 and IL-6 together with IL-23 has been advanced [117]. T_{H17} T cell subtype specifically produces IL-17A and IL-17F and following IL-23 stimulation, produces a range of inflammatory mediators including TNF- α , IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), CXCL1 and CCL20 [117-118]. It now

appears that this subset is responsible for many of the inflammatory and autoimmune responses that were once attributed to T_{H1} cells. In T1DM, the innate immune system might drive the adaptive immune system by expanding the T_{H17} T cells population. Monocytes isolated directly from the blood of T1DM patients spontaneously secreted proinflammatory cytokines IL-1b and IL-6, causing the induction of T_{H17} cells [119]. Miljkovic *et al.* demonstrated that IL-17 enhanced NO-dependent toxicity of proinflammatory cytokines toward MIN6 cells, while IL-17-specific neutralizing antibody reduced NO production partially and rescued insulinoma cells and pancreatic islets from NO-dependent damage induced by activated T cells [120]. Furthermore, a significant increase in blood IL-17 levels was observed in a multiple low-dose streptozotocin model of diabetes suggesting that the T cell-derived IL-17 might be involved in NO-dependent damage of b-cells in this disease. There is now evidence that islet-reactive T_{H17} cells promote pancreatic inflammation, but only induce insulin-dependent T1DM upon conversion into IFN- γ producers [121]. Adoptive transfer of highly purified T_{H17} cells completely devoid of IFN- γ , from BDC2.5 transgenic mice caused diabetes in immune-deficient NOD.scid recipient mice, with similar rates of onset as in transfer of Th1 cells [122]. Treatment with neutralizing IL-17-specific antibodies did not prevent disease. Instead, the transferred T_{H17} cells rapidly converted to secrete IFN- γ in the NOD.scid recipients, indicating a substantial plasticity of T_{H17} commitment toward a T_{H1}-like profile. IL-21 also appears to play a nonredundant role in autoimmunity with growing evidence of the prodiabetogenic activities of IL-21 on diverse genetic backgrounds (diabetes-prone non-obese diabetic (NOD) and C57BL/6 mice). While deficiency in IL-21R expression has been shown to render NOD mice resistant to insulinitis, insulin autoantibody production as well as the onset of T1DM, the overexpression of IL-21 in pancreatic b-cells of C57BL/6 mice induced inflammatory cytokine and chemokines, including IL-17A, IL-17F, IFN- γ , CCL2, CCL8, and interferon-inducible protein-10 (IP-10) in the pancreas. The ensuing insulinitis resulted in b-cell destruction and spontaneous T1DM in the normally diabetes-resistant C57BL/6 and NOD x C57BL/6 backgrounds [123]. A combination treatment with neutralizing anti-IL-17 antibody and recombinant IL-25 prevented the development of diabetes in NOD mice, as determined by reduced peri-islet T-cell infiltrates, decreased GAD65 autoantibody levels and increased frequency of T_{reg} cells. IL-25 treatment resulted in a T-cell-mediated dominant protective effect against autoimmunity, promoted a period of remission from new-onset diabetes and delayed recurrent autoimmunity after syngeneic islet transplantation [124]. Similarly, the combination of anti-CD154 monoclonal antibody and IL-23R antibody prevented the acute rat islet allograft rejection to some extent [125]. The pathogenic role of IL-27 in T cell-mediated autoimmune diabetes has also been demonstrated with IL-27 up-regulating pro-inflammatory cytokines IFN- γ and IL-17 and down-regulating anti-inflammatory cytokines IL-4, TGF- β and IL-10 secreted by diabetic splenocytes [126].

Conflicting results are presented for the role of NK cells in the modulation of the immune response. The immunosuppressive effects of NK cells seem attributable to TGF- β and IL-4. It has been shown that CD8⁺ T cells induced NK cells to produce TGF- β , involved in the induction of T suppressor cell activity as well as in the abrogation of immunoglobulin G synthesis [127]. In NOD mice, the adoptive transfer of diabetes into syngeneic NOD.scid recipients was prevented by complete Freund's adjuvant

(CFA) immunization and this effect was mediated by CD3⁻DX5⁺ NK cells possibly through the down-regulation of autoreactive CTL [128]. In direct contrast, it has been demonstrated that NK cells play a pivotal role in “accelerated T1DM” driven by IFN- γ . Using NOD and NOD rat insulin promoter (RIP)-IFN- γ transgenic models, Alba *et al.* demonstrated that an increase of intrapancreatic NK cells characterized the accelerated onset of diabetes accompanied by hyper-expression of pancreatic cytokines involved in NK function and migration [129]. The depletion of NK cells *in vivo* abolished the acceleration of diabetes, linking inflammation to acceleration of b-cell specific autoimmunity mediated by NK cells. Another study demonstrating that NK cells promoted the development of cyclophosphamide-induced diabetes in NOD mice involved treatment with anti-asialo-GM1 antibody [130]. The rapid IFN- γ production by NK cells could drive differentiation of naïve T cells into the T_{H1} phenotype, contributing to the autoimmune process. Also, direct interaction with APCs, such as macrophages and DCs [131] and antigen-specific T and B cells [132] might also contribute to development of the disease. A very recent study has identified NKP46, a major killer receptor expressed by NK cells that recognizes an unknown ligand expressed by β -cells and may be a new molecular target for delaying the progression of T1DM. The absence or inhibition of NKP46 prevented the development of diabetes [133].

Invariant natural killer T (iNKT) cells are a distinct line-age of innate-like T lymphocytes and converging studies using the NOD mouse model as well as the biobreeding (BB) rat model indicates reduced numbers of CD161 V α 14J α 28 and CD161⁺CD4⁺ T cells correlating with exacerbation of T1DM. Also, an increased frequency and/or activation of NKT cells prevented autoimmune diabetes [134]. Members of the NK1.1⁺ T cell family secrete high levels of IFN- γ and IL-4 after initial activation and a study indicates that the immunosuppressive activity of the bone marrow NK1.1⁺ T cells is dependent on the rapid secretion of high levels of IL-4 [135]. In NOD mice, treatment with iNKT cell activating prototypic glycolipid alpha-galactosylceramide (a-GalCer) derivative C16:0 protected them from insulinitis and T1DM [136]. In fact, several therapies that prevent T1DM in the NOD mouse model depend upon their ability to activate T_{reg} cells and/or NKT cells [105,137-139]. It is interesting to note that both cell types coexpress CD39 and CD73 and that their immunosuppressive effects are linked to the presence of adenosine [103,105]. Alterations in adenosine metabolism and CD39/CD73 adenosinergic machinery have been shown to cause loss of T_{reg} cell function and autoimmunity [140], providing new insights into the underlying mechanisms and the predisposition to autoimmunity. However, in contrast, studies by Nitta *et al.* indicate the involvement of NKT cells in both syngeneic and allogeneic immune rejection of islet allografts in the liver of mice and demonstrate the immunosuppressive role of adenosine in preventing NKT cell-mediated destruction of islet grafts [141]. Recently, a new subset, iNKT17 cells, that produce high levels of the pro-inflammatory cytokine IL-17 implicated in several autoimmune diseases, has been identified [142]. In the NOD mouse, the iNKT17 cells which are present in abundance, infiltrated the pancreas where they expressed IL-17 mRNA, enhancing the incidence of diabetes. Treatment with blocking anti-IL-17 antibody prevented disease exacerbation.

In order to further elucidate the critical role of cytokines in immune-mediated b-cell destruction leading to T1DM, Chong *et al.* overexpressed suppressor of cytokine signaling-1 (SOCS-1) and was able to completely prevent diabetes progression in CD8⁺ TCR transgenic

NOD 8.3 mice without affecting pancreas infiltration, while partially preventing disease in nontransgenic NOD mice [113]. This protection was in part due to a) inhibition of TNF- α and IFN- γ -induced Fas expression on b-cells, the expression of which was up-regulated on b-cells *in vivo* in prediabetic NOD 8.3 mice and b) inhibition of IFN- γ -induced MHC class I up-regulation as well as TNF- α - and IFN- γ -induced IL-15 expression by b-cells. Rønn *et al.* similarly demonstrated a significant resistance to cytokine-induced apoptosis and impaired insulin release in transgenic mouse/rat primary islets expressing β -cell-specific SOCS3, associated with an inhibition of IL-1-induced NF- κ B as well as MAP kinase pathways [143]. SOCS3 transgenic islets also displayed prolonged graft survival when transplanted into diabetic allogeneic BALB/c mice. Yet another study demonstrated that transplantation of chimeric adenovirus vector Ad5F35-SOCS1-infected islets into STZ-induced diabetic recipients significantly prolonged functional graft survival through caspase-3-dependent and intranuclear apoptosis inducing factor (AIF)-caspase-independent pathways [144]. Down-regulation of interferon regulatory factor-1 (IRF-1) signaling by SOCS-1 expression in b-cell also delayed islet allograft rejection [145].

Interestingly, among the regulators of apoptosis, NF- κ B has emerged as a master switch of cytokine-induced b-cell dysfunction and death [110,119]. Activation of key transcription factors such as NF- κ B and signal transducer and activator of transcription 1 (STAT-1) by activation of pattern-recognition receptors (PRRs, e.g., TLR3/4, RIGI, MDA5) on β -cells induces the release of proinflammatory chemokines and cytokines, increases MHC class I antigen expression and activates proapoptotic signals resulting in β -cell destruction. Proinflammatory cytokines further induce STAT-1, NF- κ B, and IRF-3 in β -cells, amplifying the immune processes that result in selective pancreatic β -cells destruction [110]. By employing TNF- α /IFN- γ synergism model, the antiapoptotic X-linked inhibitor of apoptosis (XIAP) molecule was shown to be upregulated by NF- κ B in response to TNF- while XIAP induction was inhibited by IFN- γ -induced STAT1 activation, which explains the death of β -cells by TNF- α /IFN- γ synergism [109,146]. In fact, Plesner *et al.* demonstrated that XIAP inhibition of β -cell apoptosis enhanced graft function and reduced the number of islets required to restore euglycemia in a syngeneic islet transplantation model promoting long-term transplant success [147].

V. STRATEGIES TO REVERSE/PREVENT TYPE 1 DIABETES MELLITUS

Currently, there exist various approaches under investigation to improve glycemic control, including amongst others, a) multiple daily insulin injections, mechanical insulin pump providing continuous insulin infusion, closed-loop insulin pumps and artificial pancreas; b) whole pancreas/pancreatic islet transplantation; c) b-progenitor cell differentiation/b-cell regeneration and expansion and d) gene therapy paradigms. These approaches broadly fall into three main categories, namely, insulin therapy or replacement with whole pancreas or islets or; regeneration of b-cell/islets from progenitors/stem cells, followed by expansion and replacement; and lastly, protection of islets from immune-mediated attack.

Restoration of Normoglycemia by Islet Transplantation

While exogenous insulin therapy remains the most common and effective standard for day-to-day control of T1DM, recurring hypoglycemic episodes [148] and microvascular

complications [149] in a high percentage of patients it necessitates whole organ transplant or pancreatic islet transplantation as a more effective treatment for the disease. Islet transplantation, whilst improving glycemic control and normalizing HbA_{1c}, is less invasive as well as hypothetically superior from a metabolic, immunologic, and patient-ease perspective [150]. Multiple centers have replicated the initial success of the Edmonton Protocol [151-153], and a 5-year post-transplant graft follow-up study indicates that the majority (~80%) of recipients were C-peptide positive post-transplant, with a 15 month median duration of insulin independence [154]. Even patients who were back on insulin but were C-peptide positive benefited from the transplantation, as shown by an HbA_{1c} of 6.7% similar to those off insulin (6.4%). The hypoglycemic score and lability index also improved significantly post islet transplantation. Similarly, one-year post-transplantation follow-up of eight female T1DM patients that received single-donor, marginal-dose islet transplants along with potent induction immunotherapy and a less diabetogenic maintenance immunosuppression, indicated 62.5% insulin independence [153]. Based on the many successful clinical islet auto- and allotransplantation trials since 1990, as well as incremental technical advances that include better identification of optimal donor characteristics, better organ retrieval techniques, minimization of cold ischemia times, better isolation technique and avoidance of immunosuppressive drugs that impair islet function and viability, human pancreatic islet allotransplantation offers a successful, effective and feasible therapeutic option in the treatment of T1DM [155-158]. In fact, islet transplantation using islets isolated from a single donor [159], as well as living donor islet transplantation to a recipient having brittle insulin-dependent diabetes with hypoglycemic unawareness have now been performed successfully [160].

While islet transplantation has become increasingly prevalent due to impressive advances in terms of improved glycemic control, relief from recurrent hypoglycemia and potential insulin independence, various challenges still preclude its wide-spread applicability. These include the limiting supply of islets for transplantation, inadequate means for preventing islet rejection and the harmful side-effects associated with chronic immunosuppressive therapy in some patients [155-157]. Additionally, while the 5-year post transplant follow-up study indicated an improvement in glycemic instability, only a minority of the patients (~10%) maintained insulin independence [154]. The 2008 Update from the Collaborative Islet Transplant Registry [161] summarizing primary efficacy and safety outcomes of 279 recipients of one or more islet alone allotransplants between 1999 and 2007, reported that 70% of islet transplant recipients achieved post-transplant insulin independence for some period of time. Of these, 71% remained insulin-independent at one year post-first infusion and 52% two years later, translating to 50% achieving and retaining insulin independence for 1 year and 35% for 2 years for all islet-alone recipients. However, at three years' post-first infusion, only 23% of recipients remained insulin independent; 29% were insulin dependent with detectable C-peptide levels; and 26% had lost all evidence of surviving islets (22% were missing data). This report also cited factors that favored the primary outcomes, including higher number of infusions, greater total number of infused islets, lower pre-transplant HbA_{1c}, islet processing at the transplant center, larger islet size and inclusion of daclizumab or etanercept during induction. Despite the less-than-optimal outcomes, an impressive improvement in metabolic control of glycemia accompanied by a clear reduction

in the occurrence of hypoglycemic episodes was observed in transplant recipients, stimulating ongoing efforts to improve long-term islet transplantation outcomes [162].

Protective Anti-inflammatory Role of A_{2A}Rs Activation in Promoting Islet Graft Survival and Reducing Islet Loss Post-transplantation

Not much is known about the immunosuppressive role of A_{2A}R activation in autoimmune diabetes. Using specific adenosine receptor agonists and antagonists, Sakowicz-Burkiewicz *et al.* demonstrated that adenosine-induced suppression of diabetic T cell proliferation was mediated by the A_{2A}R, and not the A_{2B} receptor [163]. Suppressed proliferation of diabetic T lymphocytes was evoked by the decreased expression of adenosine kinase, leading to adenosine out-flow from the cell. Extracellular adenosine then stimulated the A_{2A}R and induced cAMP production, leading to the activation of PKA and suppression of T cell proliferation. In a rat model of severe, taurocholate-induced experimental acute pancreatitis (EAP), the administration of A_{2A}R agonist CGS21680 resulted in a favorable decrease of the inflammatory cell infiltration, hemorrhagic changes, necrosis and vacuolization of acinar cells, without an evident effect on edema of the interstitial tissue [164]. These results suggest that modulation by A_{2A}R agonist could alleviate the course of severe EAP. Similarly, chronic A_{2A}R activation in diabetic rats ameliorated histological and functional changes in kidneys induced by diabetes and reduced inflammation associated with diabetic nephropathy [45]. An upregulation in A_{2A}R expression in hippocampus as well as in whole heart has been observed in streptozotocin induced diabetic rats [165]. Interestingly Nemeth *et al.* demonstrated that nonselective adenosine receptor agonist NECA prevented diabetes development in both, multiple low-dose, streptozotocin-challenged mice as well as in cyclophosphamide-treated NOD mice. This protective effect was at least in part mediated by A_{2B}R, since the protective effect could be reversed using a selective A_{2B}R antagonist [20].

A recent study by Zarek *et al.* demonstrating that A_{2A}R agonist administration in mice not only inhibited T_{H1} and T_{H17} effector cell generation but also promoted the generation of adaptive T_{reg} cells is of tremendous significance, given the involvement of these subsets in transplant rejection and tolerance respectively [166]. In their investigation of the effect of adenosine therapy in islet transplantation, Nitta *et al.* demonstrated that a single dose of adenosine delivered at the time of islet transplantation suppressed NKT cell-mediated IFN- γ production by Gr-1⁺CD11b⁺ cells (neutrophils) in the liver of streptozotocin-induced diabetic recipient mice, thus preventing early loss of transplanted syngeneic and allogeneic islets and enabling transplantation using a single donor [141]. This observation combined with the observation of Lappas *et al.* that A_{2A}R agonists were able to suppress hepatic IRI by inhibiting activation of NKT cells positively indicates the involvement of NKT cells in the early loss of transplanted islets. Whether the mechanism of inhibition lies upstream of NKT cell activation, with an involvement of dendritic and kupffer cells, is yet to be elucidated. In an attempt to further determine the extent and the efficiency of improvement in islet transplantation by adenosine administration, an intra-peritoneal glucose tolerance test (IPGTT) performed 60 days post-transplantation showed that the glucose tolerance of diabetic mice receiving 200 islets from a single donor and treated once with adenosine was superior in comparison with diabetic mice receiving 400 islets from two donors, indicating that adenosine facilitated a greater than 2-fold improvement in the efficiency of islet

transplantation. The beneficial effect of adenosine therapy was also observed in islet allotransplantation when treatment included an immunosuppressive agent such as anti-CD4 antibody. This study supports an earlier observation by Yasunami *et al.* that survival of islet allograft in the liver of mice was prolonged without any immunosuppression in Va14 NKT cell- and CD1d-deficient mice [167], indicating an essential role for NKT cells in alloimmune rejection of islets [167-168]. Interestingly, Satoh *et al.* also showed that successful islet transplantation from one donor to two recipients was feasible by targeting pro-inflammatory cytokines in mice: restoration of normoglycemia was achieved in WT diabetic mice receiving 100 syngeneic islets graft by administration of anti-TNF- α and anti-IL-1 β antibodies in conjunction with anti-IFN- γ antibody [169]. Transplantation of 200 syngeneic islets from a single donor pancreas into IFN- $\gamma^{-/-}$ mice produced similar results. Normoglycemia was also attained by the combined use of antibodies in mice receiving islet allografts when rejection was prevented with anti-CD4 antibody treatment. However, the role of A_{2A}R agonists-mediated inhibition of the cytotoxic activity and cytokine production by activated NKT in islet transplantation is yet to be elucidated. A recent study by Ghaemi Oskouie *et al.* demonstrated that upregulation and subsequent activation of adenosine deaminase in NOD DCs triggered autoimmune T cell activation and diabetes in NOD mice [170]. DCs deficient in ADA expression have a greatly reduced capacity to trigger T1DM.

Our studies investigating the effect of ATL146e or ATL313 administration on syngeneic islet transplantation indicate that administration of A_{2A}R agonists improves *in vitro* glucose stimulated insulin secretion (GSIS) by an effect on leukocytes (PMNLs) as well as survival and functional engraftment of transplanted islets by inhibiting inflammatory islet damage in the peritransplant period [171]. In *in vitro* studies, islets that were co-cultured with syngeneic leukocytes and challenged by GSIS demonstrated an inhibition of the islet stimulation index in the presence of leukocytes. Addition of ATL146e to culture media preserved islet insulin secretory capacity in the islet/leukocyte cocultures and restored stimulation index to control values. The beneficial effect was blocked by addition of the A_{2A}R selective antagonist SCH58261. The hypothesis that ATL/A_{2A}R binding on inflammatory cells would inhibit inflammation-mediated islet cell damage and dysfunction was further supported by the observed failure of ATL146e to protect *in vitro* insulin secretion by islets co-cultured with A_{2A}R^{-/-} leukocytes, suggesting limited endogenous protection in this model. In histological studies, islets were transplanted under the renal subcapsule of diabetic C57BL6 syngeneic recipients, with or without ATL therapy, and islet graft-bearing kidneys were harvested at specific time points post-transplant. Histological evaluation indicated the presence of infiltrating neutrophils in islet graft biopsies 24 hours following transplantation, with lymphocytes and lymph nodules evident by post-transplant day 5 in recipients that did not receive ATL therapy. A_{2A}R agonist treatment demonstrated marked reductions in neutrophils and lymphocyte infiltration, reduced cellular necrosis and fibrosis as well as a reduction in the size and number of lymphoid nodules. *In vivo* studies indicated that streptozotocin-induced diabetic recipient C57BL/6 mice receiving islet transplants to either the renal subcapsular, or hepatic-intraportal site achieved normoglycemia more rapidly with ATL therapy than untreated recipients. Interestingly, transplantation of the minimal threshold dose of islets to diabetic mice dosed with ATL146e, beginning posttrans-plant day -3, resulted in normoglycemia in all mice within 5 days of

transplantation compared to diabetic mice dosed with ATL146e beginning posttransplant day -1 wherein only a transient improvement in glycemic status was observed, suggesting at least 72 hours of ATL146e therapy is required to achieve full anti-inflammatory efficacy. No significant temporal improvement in graft outcome (reduced time to normoglycemia) was observed between C57BL/6 recipients of syngeneic islets and $A_{2A}R^{-/-}$ recipients of congenic C57BL/6 islets as well as no difference was observed between $A_{2A}R^{-/-}$ recipients of congenic C57BL/6 islets with or without administration of ATL146e therapy.

VI. FUTURE TRENDS AND CONCLUSION

The therapeutic utility of $A_{2A}R$ s agonists as an anti-inflammatory and immunosuppressive agent in IRI as well as in several other diseases is well established. However, the wide spread tissue/cell-type distribution of $A_{2A}R$ s could potentially lead to unwanted side-effects upon receptor activation. Additionally, the ability of $A_{2A}R$ agonists to promote profound vasodilation is probably another reason that has prevented A_{2A} receptor agonists from being adequately tested in clinical trials as an anti-inflammatory drug. In order for $A_{2A}R$ agonists to be successfully utilized in islet transplantation, it is necessary to develop strategies that would target the action of $A_{2A}R$ agonists to the graft site in order to avoid systemic side-effects. For e.g., placement of absorbable biomatrix scaffolds engineered to contain $A_{2A}R$ agonists at the graft site would help modulate the immune environment in the immediate vicinity of the graft such that innate inflammatory response and loss of islets is reduced. Our preliminary studies using poly(DL-lactide-co-glycolide) electrospun scaffolds loaded with sphingosine 1 phosphate pro-drug FTY720 to drive islet revascularization post transplantation, significantly increased microvessel length density over 7 days in streptozotocin-induced diabetic C57b16/J mice [172]. Studies with $A_{2A}R$ agonists are currently underway. The ability of $A_{2A}R$ agonists to promote proliferation and differentiation of mouse bone marrow mesenchymal stem cells (BM-MSCs) opens another avenue for the utilization of $A_{2A}R$ agonists in the field of insulin producing cell regeneration and expansion for islet transplantation [173]. Several studies have demonstrated the capacity of BMMSCs to generate insulin producing cells and abrogate immune injury [156,158,174]. These insulin producing cells expressed significant levels of pdx1, insulin, and glucagon and released insulin in a glucose-dependent manner that led to amelioration of diabetic conditions in streptozotocin-treated diabetic mice. Furthermore, MSC-islet cotransplantation also improved islet graft function and survival in diabetic rats by promoting graft revascularization, suppressing inflammation and maintaining islet organization and morphology [175-176]. Therefore, the observation that adenosine and its A_{2A} receptor play a critical role in promoting the proliferation and differentiation of mouse BM-MSCs is of tremendous significance in treatment of T1DM.

Our studies indicating that administration of $A_{2A}R$ agonist in the peritransplant period reduced inflammation-mediated early cell loss in syngeneic islet transplantation, thereby allowing for an accelerated rate of cure with a smaller number of donor islets both in the kidney subcapsule transplant model as well as a clinically-relevant intrahepatic portal vein model, suggests a potentially significant new strategy for reducing inflammatory islet loss in clinical transplantation. In islet transplant protocols which minimize systemic immunosuppression, including a short course of an $A_{2A}R$ agonist as part of induction

therapy could minimize early peritransplant islet loss and reduce the number of islets required for successful clinical islet transplantation, thus increasing the efficacy of the procedure and increasing the availability of this procedure to a greater number of potential recipients. Future studies delineating the molecular mechanism of A_{2A}R activation, i.e. the immune cells and the cytokine and chemokine mediators involved in mediating the anti-inflammatory effect of A_{2A}R agonists in murine islet allotransplantation will help evaluate the benefit of using anti-inflammatory agonists as therapeutic adjuncts to the immunosuppressive regimens used in clinical islet transplantation.

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