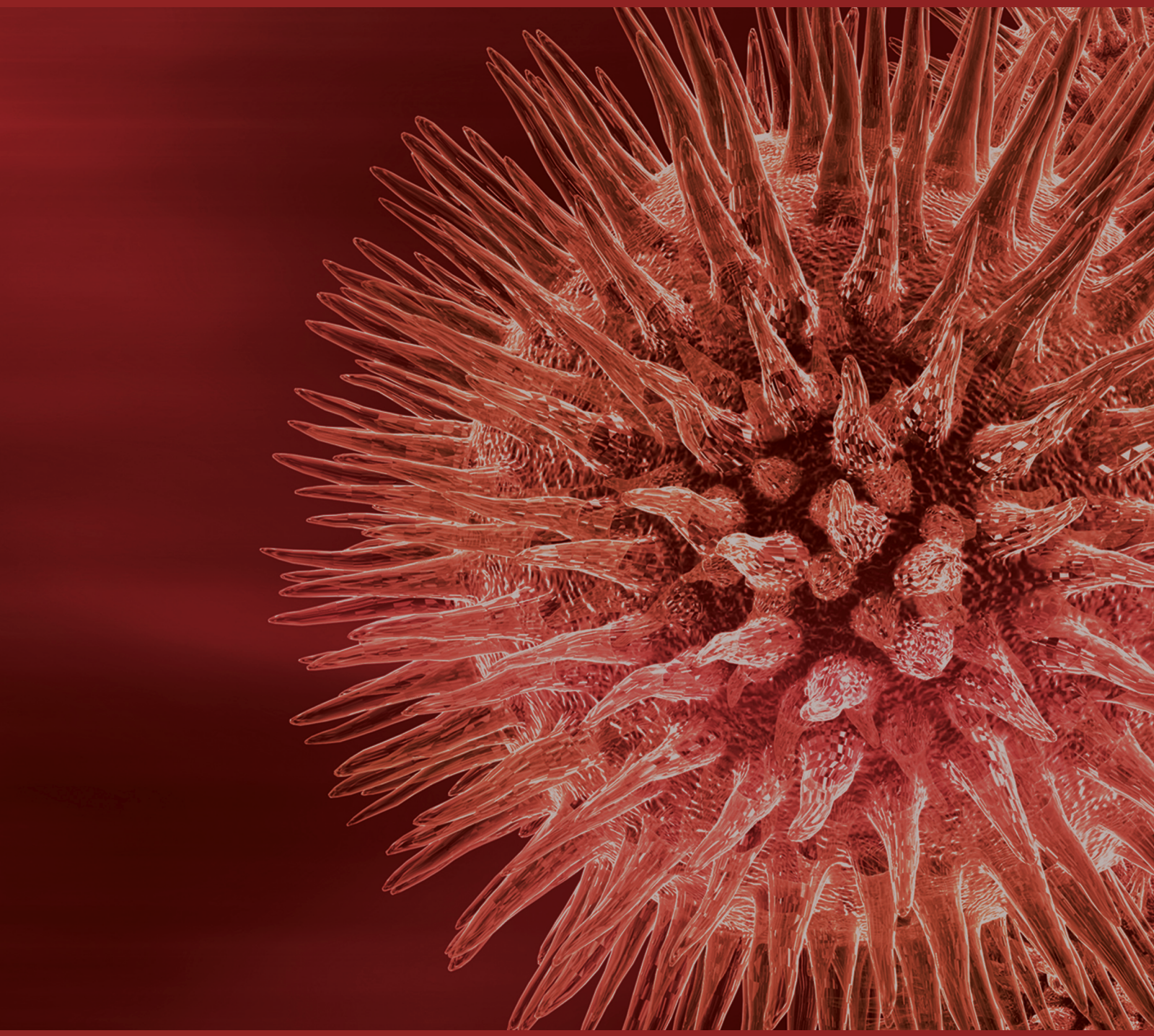
Journal of Biomedicine and Biotechnology

Ectonucleotidases in Cancer and Inflammation

Guest Editors: John Stagg, Linda F. Thompson, and Karen M. Dwyer



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Editorial

Ectonucleotidases in Cancer and Inflammation

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In 1970, Geoffrey Burnstock described the release of extracellular adenosine triphosphate (ATP) as a transmitter substance by nonadrenergic inhibitory nerves, and later in 1972, he formulated the hypothesis of purinergic neurotransmission [1]. Together with Che Su and John Bevan, Geoffrey Burnstock built the foundation to what was to become an entirely new field of research in biology [2]. While early studies focused on the role of purinergic receptors in neurotransmission, it soon became obvious that extracellular ATP and its hydrolyzed derivative adenosine had important roles in immune regulation. It is now recognized that purinergic signaling not only regulates neurotransmission and inflammation, but also influences diverse biological pathways, such as cell survival, proliferation, differentiation, lipid synthesis, and cell motility.

In 1978, Burnstock proposed two types of purinergic receptors: P1 receptors selective for adenosine and P2 receptors selective for ATP and ADP. In 1985, a pharmacological approach was proposed to distinguish between two types of P2 receptors: ionotropic P2X and metabotropic P2Y receptors [3]. Nucleotides, such as ATP, are released by a variety of cell types especially under stress conditions. Cancer cells, for instance, are known to release ATP in the tumor microenvironment. Nucleotides that are released in response to a stress signal can be hydrolyzed by membrane-bound enzymes called ectonucleotidases. By regulating the levels of extracellular nucleotides and nucleosides, ectonucleotidases are thus involved in numerous physiological and pathological conditions.

In this special issue, P. Chernogorova and R. Zeiser discuss the role of ectonucleotidases, in particular CD39 (NTPDase1) and CD73 (ecto-5′-nucleotidase) on allograft rejection, acute graft-versus-host disease (GvHD), and graft-versus-leukemia (GvL) effect. Interestingly, recent evidence suggests that purinergic signaling influences the severity of alloimmune responses. As highlighted by P. Chernogorova and R. Zeiser, these studies suggest potential clinical use of recombinant ectonucleotidases or adenosine receptor agonists for regulation of alloimmune responses which can be tailored according to the clinical situation.

F. Ghiringhelli et al. review the current literature on the role of ectonucleotidases in tumor immune escape, with a focus on the immune regulatory function of Th17 cells. Th17 cells have emerged as key participants in a wide range of immune disorders and cancers. While the role of Th17 cells in cancer immunity remains controversial, F. Ghiringhelli et al. describe their recent seminal observation that ectonucleotidases are expressed on Th17 cells and that this is relevant in the context of tumor growth.

A. Battastini and colleagues present an overview of the various roles of purinergic signaling in gliomas. In gliomas, the presence of an inflammatory infiltrate is directly correlated with tumor malignancy, and this appears to be regulated in part by purinergic signaling. The authors discuss the observation that ATP triggers glioma cells to release proinflammatory factors important for the recruitment of monocytes and neutrophils, thereby favoring tumor growth. The authors discuss their own work that aims to establish whether ectonucleotidases are involved in macrophage polarization in gliomas.

A. Czopik and colleagues present a research article describing the protective role of CD73 in a model of colitis

induced by administration of dextran sulfate sodium salt (DSS). The authors demonstrate that compared to wild-type mice, CD73-deficient gene-targeted mice are highly susceptible to DSS-induced colitis. The authors conclude that CD73 expression in the colon is critical for regulating the magnitude and the resolution of colonic immune responses.

K. M. Dwyer and colleagues discuss the role of the CD39-adenosinergic axis in the pathogenesis of type 1 and type 2 diabetes. They discuss recent work suggesting that CD39 is involved in the pathophysiology of pancreatic dysfunction. This has importance clinical consequences, as drug development targeting different components of the pathway may be of relevance in the treatment of both type 1 and type 2 diabetes. The authors also discuss unanswered questions, such as what is the source of ecto-5′-nucleotidase activity given the lack of CD73 expression within the pancreas.

J. Stagg and colleagues describe the role of CD73 and extracellular adenosine signaling in promoting tumor growth through paracrine and autocrine action. The authors discuss their recent work on the role of CD73 in spontaneous tumor growth and on the role of CD73 and adenosine on endothelial and immune cells.

In conclusion, this special issue on ectonucleotidases aims to acquaint investigators not familiar with this field with important recent advances with the hope of attracting scientists to join the effort of exploiting modulation of purinergic signaling for therapeutic purposes to treat cancer and inflammatory diseases.

John Stagg
Linda F. Thompson
Karen M. Dwyer

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Review Article

CD73-Generated Adenosine: Orchestrating the Tumor-Stroma Interplay to Promote Cancer Growth

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Despite the coming of age of cancer immunotherapy, clinical benefits are still modest. An important barrier to successful cancer immunotherapy is that tumors employ a number of mechanisms to facilitate immune escape, including the production of anti-inflammatory cytokines, the recruitment of regulatory immune subsets, and the production of immunosuppressive metabolites. Significant therapeutic opportunity exists in targeting these immunosuppressive pathways. One such immunosuppressive pathway is the production of extracellular adenosine by CD73, an ectonucleotidase overexpressed in various types of cancer. We hereafter review the biology of CD73 and its role in cancer progression and metastasis. We describe the role of extracellular adenosine in promoting tumor growth through paracrine and autocrine action on tumor cells, endothelial cells, and immune cells.

1. Cancer Immunotherapy: An Overview

The recent FDA approval of ipilimumab (Yervoy, Bristol-Myers Squibb)—an antibody that blocks the inhibitory T cell receptor CTLA-4—for treatment of metastatic melanoma and sipuleucel-T (Provenge, Dendreon)—a cell-based vaccine—for treatment of castration-resistant prostate cancer, has revitalized the interest for cancer immunotherapy [1]. The enthusiasm generated by these new treatments is further fuelled by overwhelming new data revealing the importance of tumor immune infiltrates in the survival of cancer patients. Indeed, the presence of CD8+CD45RO+ T cells in tumors is associated with a good prognosis in various types of epithelial cancers [2]. In cancers such as colorectal cancers, T-cell infiltration has in fact superior prognostic power than standard staging methods [3].

In addition of being involved in the natural progression of cancer, immune responses affect the activity of anticancer treatments [4]. Accordingly, recent studies revealed that some chemotherapeutic drugs, such as anthracyclines and oxaliplatin, specifically rely on the induction of anticancer immune responses for therapeutic activity [5]. Immune responses also play a major role in the efficacy of targeted therapies with monoclonal antibodies (mAbs). While antibody-dependent cellular cytotoxicity (ADCC) is important in the activity of tumor-targeted mAb therapies, recent studies suggest that mAbs such as trastuzumab may also stimulate adaptive antitumor immunity [6]. Taken together, this suggests that incorporating immunotherapeutic approaches to standard treatments might in fact be synergistic.

Much of the recent successes in cancer immunotherapy come from blocking mAbs targeting immune checkpoint inhibitors, such as CTLA-4 and PD-1. In 2011, the FDA approved the use of the anti-CTLA-4 mAb ipilimumab in patients with metastatic melanoma. However, one of the drawbacks to anti-CTLA-4 mAb therapy is the generation of autoimmune toxicities due to on-target effects. Accordingly, it has been reported that up to 23% of patients treated with ipilimumab developed serious grade 3-4 adverse events [7]. Another promising form of cancer immunotherapy consists of blocking mAbs against PD-1 or its ligand PD-L1. Administration of anti-PD-1 or anti-PD-L1 mAb enhances adaptive anti-tumor immune responses by preventing T-cell exhaustion. In early clinical trials, both anti-PD-1 and anti-PD-L1 mAbs have shown impressive objective responses

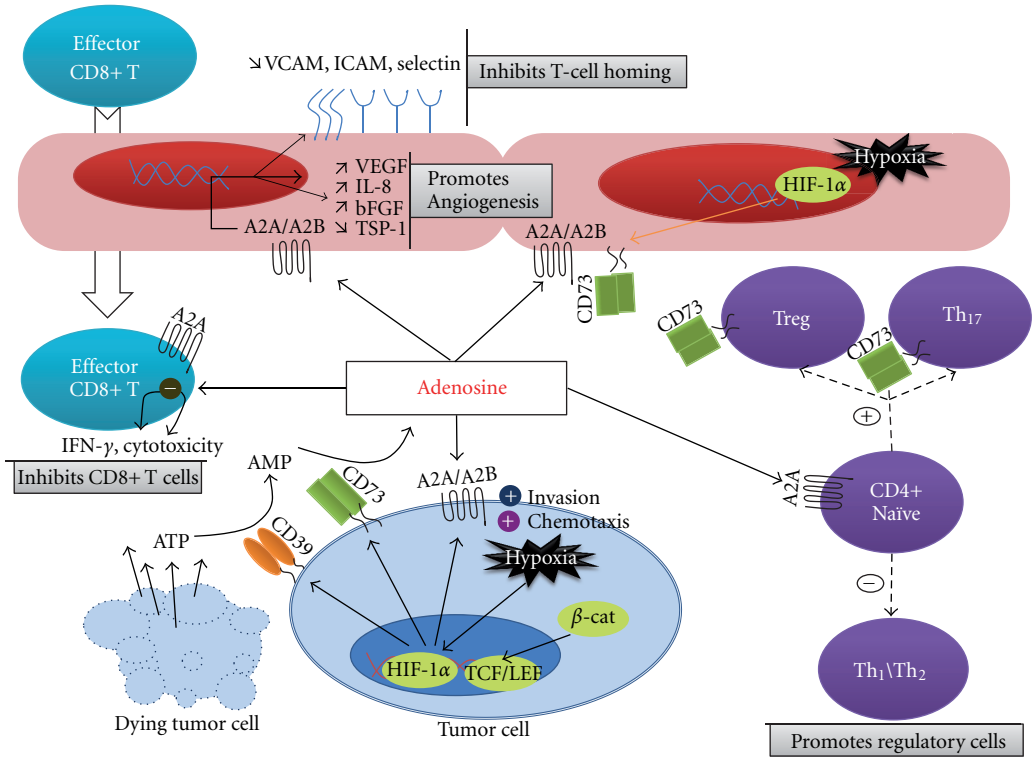

Figure 1: CD73-generated adenosine orchestrates the tumor-stroma interplay to promote cancer growth. The concerted action of CD39 and CD73 represents the main pathway for extracellular adenosine production in the tumor microenvironment. These two ectonucleotidases are expressed not only by tumor stromal cells (such as endothelial cells or tumor-associated regulatory T cells) but also by certain cancer cells, allowing for the conversion of extracellular ATP (released by dying tumor cells) into adenosine. Adenosine exerts its tumor-promoting effects in paracrine and autocrine fashion by activating adenosine receptors expressed by tumors cells, endothelial cells, or immune cells. Activation of A2A adenosine receptors inhibits IFN-γ production and cytotoxic killing by CD8+ T cells and promotes CD4+ cells differentiation into T-regulatory cells. This immunosupressive effect is strengthened by adenosine action on the tumor-surrounding endothelium which consists in repressing T-cell homing to tumors through the downmodulation of adhesion proteins such as ICAM-1, VCAM-1 or P-selectin. Simultaneously, A2A and A2B engagement on endothelial cells also enhance the production of proangiogenic factors including VEGF, b-FGF, and IL-8. This effect is mediated by HIF-1 and synergizes with the hypoxic tumoral microenvironment. Finally, CD73-generated adenosine also promotes tumor development by directly acting on cancer cells through A2A and/or A2B adenosine receptor activation and subsequent enhancement of invasiveness and chemotactic response.

in patients with nonsmall-cell lung cancer, melanoma, and renal-cell cancer [8, 9].

Despite this coming of age of cancer immunotherapy, clinical benefits are still modest. One potential explanation is that tumors employ a number of mechanisms to facilitate immune escape, including the production of anti-inflammatory cytokines, the recruitment of regulatory immune subsets, and the production of immunosuppressive metabolites. Significant therapeutic opportunity exists in targeting these immunosuppressive pathways. One such therapeutic target is CD73, an ectoenzyme that catalyses the generation of extracellular adenosine, a potent immunosuppressive molecule. We hereafter review the biology of CD73 and its role in cancer progression and metastasis.

2. CD73 Biology and the Adenosinergic Signaling

CD73 is a glycosylphosphatidylinositol (GPI-) anchored nucleotidase present in cell membrane lipid rafts, active as a disulfide-linked homodimer, which catalyses the hydrolysis of extracellular adenosine monophosphate (AMP) into adenosine [10]. CD73 is expressed on lymphocytes, endothelial and epithelial cells, where it participates in ion transport regulation, endothelial cell barrier function, endothelial homeostasis, and protection from ischaemia [11–13].

CD73 also has a predominant role in immunity (Figure 1). Indeed, CD73 negatively regulates the proinflammatory effects of extracellular adenosine triphosphate (ATP). Extracellular ATP, released by damaged or dying cells and bacteria, promotes the recruitment of immune phagocytes [14] and activates P2X7R, a coactivator of the NLRP3 inflammasome, which then triggers the production of proinflammatory cytokines, such as IL-1β and IL-18 [15]. The catabolism of extracellular ATP into ADP, AMP and adenosine is controlled by ectonucleotidases and membrane-bound kinases. Whilst hydrolysis of ATP into AMP is predominantly performed by CD39 (ENTPD1), CD73 catalyses the conversion of AMP into adenosine. Hence, CD39 and CD73 act in concert to convert proinflammatory ATP into

immunosuppressive adenosine. Importantly, the activity of CD39 is reversible by the actions of NDP kinase and adenylate kinase, whereas the activity of CD73 is virtually irreversible. Thus, CD73 represents a crucial checkpoint in the conversion of proinflammatory ATP into immunosuppressive adenosine.

To mediate its physiological actions, CD73-generated adenosine can bind to four distinct G-protein-coupled receptors: A1, A2A, A2B, and A3 [16]. The A1 and A3 adenosine receptors are coupled with the $G_{i/o}$ subunit, which leads to the inhibition of adenylyl cyclase, cyclic AMP (cAMP) production, and protein kinase A (PKA) activation. A1 and A3 receptors have also been linked to the activation of the phosphatidylinositol 3-kinase (PI3 K) pathway. In contrast, A2A and A2B are coupled with the G_s subunit that stimulates cAMP production, and PKA activation. All four adenosine receptors have been associated with the activation of the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) pathways.

3. Expression of CD73 on Cancer Cells

CD73 has been found to be overexpressed in several types of cancer, including bladder cancer, leukemia, glioma, glioblastoma, melanoma, ovarian cancer, colon cancer, and breast cancer [17–20]. This overexpression can be explained by several mechanisms. In the context of breast cancer, loss of estrogen receptor (ER) expression has been shown to induce constitutive CD73 expression [20]. Thus, CD73 is highly expressed in ER-negative breast cancer cells and might constitute a promising target for treatment-refractory breast tumors such as ER-negative or triple-negative breast cancer [21].

Another factor that can drive CD73 expression is the hypoxic nature of the tumor microenvironment. Indeed, the CD73 gene promoter has at least one binding site for the hypoxia-inducible factor (HIF-) 1α and, not surprisingly, hypoxia promptly stimulates CD73 expression. Inhibition of HIF-1α by antisense oligonucleotides or point mutations in the hypoxia response element of the CD73 promoter has been reported to inhibit hypoxia-induced CD73 expression [22–24]. Notably, hypoxia also upregulates CD39, A2A, and A2B adenosine receptor expression [25, 26].

Another pathway that can induce CD73 expression is the Wnt pathway. Accordingly, a TCF/LEF consensus binding site is present in the CD73 gene promoter and it has been shown that Wnt signaling can drive CD73 expression [27, 28]. Of interest, the Wnt pathway is often deregulated in human tumors by the loss of the tumor suppressor APC or by mutations in the β-catenin gene [29].

CD73 expression is also regulated epigenetically. Wang et al. recently reported that CD73 expression is downregulated by methylation-dependent transcriptional silencing in several human melanoma cell lines [30]. Notably, relapse with metastatic disease was found to be more frequent in melanoma patients lacking CD73 methylation. While this observation must be validated in a larger cohort, it supports the notion that CD73 might constitute a valid therapeutic target in melanoma.

Finally, CD73 overexpression on tumors may also result from a selective pressure exerted by the immune system. Indeed CD73 positive tumor cells, via the production of immunosuppressive adenosine, are better equipped to evade anti-tumor immune responses.

4. Prognostic Implications of CD73 Expression

Despite the fact that CD73 expression has been observed in several types of cancer, correlative analysis to clinical outcome has been relatively limited [31]. Recently, Wu et al. evaluated the prognostic relevance of CD73 in colorectal cancer (CRC): analysis of CD73 expression by immunohistochemistry (IHC) revealed that high levels of CD73 were correlated with a poor prognosis ($n = 342$ patients) [32]. In breast cancer, one study suggested a negative correlation between ER and CD73 expression ($n = 18$ patients) [20] and a retrospective analysis of 30 breast cancer biopsies found that CD73 expression was associated with an increased risk of relapse and increased likelihood of metastasis [33]. In contrast, another recent study reported that high CD73 expression levels were associated with a good prognosis in breast cancer patients [34]. The prognostic implication of CD73 expression in breast cancer thus remains controversial.

A recent study assessed the clinical implication of CD73 in chronic lymphoblastic leukemia (CLL) [17]. The study evaluated CD39 and CD73 expression on 299 blood samples and found that all CLL samples expressed CD39 while only one third expressed CD73. CD73 expression was found to be highest on CD38+, ZAP-70+, or Ki-67+ leukemic cells, suggesting that CD73 was associated with a more aggressive and proliferative disease. CD73 was found to be particularly abundant in lymph nodes, on highly proliferating cells and in perivascular areas suggesting a role in lymph node homing. The authors also identified a significant elevation of A2A adenosine receptor expression in leukemic patients compared to healthy controls. Notably, A2A activation on CLL cells was linked to an increase resistance to drug-induced apoptosis. Taken together, these results suggest that adenosine is part of an autocrine/paracrine loop that enhances CLL cells chemoresistance and favours their arrest in lymph node proliferation centers [17].

5. CD73 Expression on Regulatory T Cells

T-regulatory cells (Tregs) are naturally occurring or inducible T cells specialized in suppression of immune responses. Treg-mediated immunosuppression can operate through several mechanisms, including CD73-mediated production of extracellular adenosine, which has recently emerged as a key process implemented by Tregs and exploited by various tumors to dampen immune responses [31].

CD73 is expressed on different subsets of T lymphocytes, but it is particularly abundant in Foxp3+ Tregs (37). In mice, CD73 is expressed in approximately 60% of CD4+ and 80% of CD8+ T cells and is predominant in $CD25^{high}$/CD39+/Foxp3+ T cells [35–37]. The coexpression of CD73 and CD39 on Treg provides them with the complete enzymatic machinery to produce immunosuppressive

adenosine. In humans, CD73 expression in T cells is more limited, with CD73 expression highly correlated to the presence of CD25, CD39, and Foxp3 on CD4 T cells [38]. Amongst human Tregs, 70–80% coexpresses CD73 and CD39 [38]. In human Tregs, CD73 appears to be stored intracellularly [38], which may account for previous reports pointing out to a large discrepancy concerning CD73 expression in mouse and human Tregs [39, 40]. CD73 expression on Tregs is modulated upon activation and depends on the cytokine milieu. Indeed, it has been shown that CD73 expression is augmented upon TCR engagement concomitantly to P2X7 receptor downmodulation, thus favoring the production of immunosuppresive adenosine and limiting proapoptotic effects of ATP [41]. Another study has demonstrated that CD73 expression is enhanced when CD4+ cells are activated in the presence of TGF-β [36]. This effect has been observed in both CD4+/Foxp3+ and CD4+/Foxp3− cells, confirming that CD73 upregulation can occur independently of Foxp3. A recent publication confirmed these results and showed that both TGF-β and IL-6 are required for CD73 and CD39 expression on Th17 cells [42]. Signaling pathways investigation revealed that TGF-β-induced inhibition of Gfi-1 transcription repressor coupled to IL-6-induced STAT-3 activation was necessary for ectonucleotidase expression on Th17 cells. In several murine cancer models where TGF-β accumulates in the tumor microenvironment, CD39 and CD73 expression on Th17 cells actively participates in tumor immunoevasion [42].

CD73-mediated production of adenosine is thus crucial for Tregs immunosuppressive potential [35]. This has been revealed by an impaired immunosuppressive potential of Tregs derived from CD39- or CD73-deficient mice [35, 41]. In CD73-deficient mice, activated CD4+ cells showed an augmented production of proinflammatory cytokines (IFN-γ, IL-2, and TNF-α) [41] and loss of immunosuppressive function of Tregs. In CD39-deficient mice, Tregs were shown to be constitutively activated, thus abrogating their suppressive activity on non-Tregs cells [35]. Taken together, these studies demonstrated that Tregs immunosuppression relies, at least in part, on the hydrolysis of extracellular ATP into adenosine by the concerted action of CD39 and CD73 and the subsequent engagement of A2A on effector T cells. A2A activation on T-effector cells induces a long-term anergy, characterized by an impaired proliferation upon TCR engagement and a reduction in proinflammatory cytokines production [43]. Moreover, this anergy-like state cannot be reversed by TCR reengagement, even in the absence of A2A signaling. Complementary *in vivo* experiments, using a model of lung autoimmunity, showed that A2A activation is involved in Th17 cells inhibition (via the inhibition of IL-6 synthesis) and in the induction of immunosuppressive LAG3+ Treg cells [43].

The importance of CD73 expression on Tregs has also been documented in various tumor models [44, 45]. Using DEREG transgenic mice (expressing the diphtheria toxin receptor under the control of Foxp3) adoptively reconstituted with Tregs from CD73-deficient or wild type mice, Stagg et al. demonstrated that CD73 expression on Tregs is crucial to promote the growth of MC38 colon tumors [44]. Another independent research group obtained similar results with B16F10 tumors implanted in RAG1−/− mice adoptively transferred with CD73-deficient or wild type T cells, with or without depletion of CD25+ T cells [45, 46]. These studies highlighting the role of CD73 on mouse Tregs have been corroborated by observations made in human Tregs. Accordingly, analysis of PBMC of cancer patients revealed that the expression and frequency of CD73+ Tregs are elevated compared to healthy volunteers [47]. Moreover, Tregs obtained from cancerous patients have increased nucleotidase activity, increased suppressor functions, and are able to infiltrate tumors [47]. Interestingly, complementary *in vitro* studies pointed out to a synergistic effect of adenosine and prostaglandin E2 (PGE2) in mediating Treg suppression [48]. Notably, COX2+ tumor cells were demonstrated to promote adenosine and PGE2 synthesis by Tregs in coculture experiments.

6. CD73 Expression on Endothelial Cells

Vascular and lymphatic endotheliums are crucial organs for leukocytes trafficking into tissues. The selective permeability and barrier function of the endothelium are tightly controlled and dysregulation of endothelial barrier hemostasis is associated with several diseases, including cancer.

CD73 and adenosine receptors are important regulators of leucocyte trafficking and endothelial hemostasis [49]. CD73 possesses distinct roles on lymphatic and vascular endothelium [50]. On vascular endothelial cells, CD73 participates in leukocytes extravasation from blood. It has been shown that leukocyte binding on endothelial cell triggers the inhibition of CD73 nucleotidase activity, thereby reducing local adenosine production which favors vascular permeability and leukocyte transmigration [12]. Leukocyte CD73 instead contributes to trafficking across lymphatics [50]. The importance of CD73 on endothelial cell function has also been largely evidenced by the analysis of CD73 deficient mice [51]. CD73-deficient mice present elevated expression of VCAM-1 in carotid arteries associated with an increased accumulation of monocytes [52]. Unexpectedly, in experimental autoimmune encephalomyelitis (EAE, a model of multiple sclerosis), CD73-deficiency resulted in resistance to the disease [53]. This resistance is thought to be the result of a lack of lymphocyte infiltration in the central nervous system. Interestingly, A2A blockade protected wild type mice against EAE and was associated with a downregulation of ICAM-1 expression on the choroid plexus.

In humans, a recent study identified mutations in the CD73 gene resulting in a nonfunctional protein and the development of symptomatic arterial and joint calcification, a pathology associated with an excess risk of cardiovascular events. The increase in ectopic tissue calcification associated with a non-functional CD73 protein was found to be dependent on an increase in tissue nonspecific alkaline phosphatase (TNAP). Therefore, targeted blockade of CD73 as a therapeutic approach could theoretically be combined with inhibitors of TNAP such as bisphosphonates or lansoprazole in order to prevent the risk of arterial calcification [54].

Activation of the adenosinergic system on endothelial cells is largely regulated by hypoxia. A2B adenosine receptor and neutrophils play a central role in promoting endothelial cell barrier function during hypoxia [25, 55, 56]. Hypoxia upregulates A2B expression via HIF-1α [57] and activation of A2B potentiates the secretion of VEGF and other proangiogenic factors such as IL-8 [58] or basic fibroblast growth factor (bFGF) [59]. Hypoxia further drives expression of A2A via HIF-2α. A2A activation in endothelial cells has been to shown enhance proliferation, migration, and capillary-like tube formation [60]. Interestingly, A2A-mediated tube formation was shown to depend on the inhibition of the antiangiogenic factor thrombospondin-1 [61]. Adenosine receptor activation on endothelial cells was also demonstrated to enhance VE-cadherin expression in a A2A, dependent manner, P-selectin expression via A2B and CD73 expression in a A2B dependent fashion [58, 62, 63].

In addition to hypoxia, CD73 and adenosine receptors have been shown to be regulated by cytokines and growth factors. For instance, types I and II IFNs increase CD73 expression at the surface of endothelial cells, thus promoting adenosine generation and subsequent enhancement of endothelial cell barrier function [64, 65]. Likewise, A2A and A2B expressions are augmented on endothelial cells upon IL-1 or TNF-α stimulation [66]. Surprisingly, TNF-α does not upregulate CD73 but, on the contrary, reduces CD73 nucleotidase activity by triggering CD73 shedding from plasma membrane in a PLC-dependent manner [67]. Interestingly, CD73 shedding has been described on lymphocytes upon CD73 engagement by a specific monoclonal antibody and was associated with enhanced lymphocyte adhesion to endothelial cells through a calpain-like enzyme-mediated LFA-I clustering [68, 69]. In contrast, endothelial CD73 engagement does not entail its cleavage from plasma membrane.

7. CD73 and Tumor Metastasis

Tumor metastasis is a complex multistep process associated with poor prognosis [70]. Therefore, identification of metastasis-promoting pathways is of primary importance for the development of new anticancer treatments. In this regard, accumulating data indicate that CD73 promotes tumor metastasis. Two independent studies have correlated CD73 expression with lymph node metastasis of breast tumors [33, 71]. In both studies, relevance of the results obtained with cell lines and murine models was confirmed in patient biopsies, revealing that CD73 is upregulated in metastatic tumors, lymph node foci and associated with disease relapse [33]. CD73 upregulation in highly metastatic breast tumor cells might be associated with a loss of CpG island methylation in the NT5E gene, as recently observed by Wang et al. [30].

CD73 implication in tumor metastasis has been evidenced in our recent studies using CD73-null mice. We showed that CD73-deficient mice are resistant to experimental lung metastasis following intravenous injection of B16F10 melanoma cells or TRAMP-C1 prostate cancer cells [44, 72]. Interestingly, the resistance of CD73 KO mice to experimental metastasis was independent of the immune system. This observation infers that CD73 expression on non-hematopoietic cells, presumably endothelial cells, promotes the metastatic process. CD73 expression on tumor cells can also promote tumor metastasis in mice, most likely via an autocrine activation of A2B adenosine receptor [73]. This is concordant with data obtained by other groups who reported that CD73 overexpression or adenosine receptors activation on cancer cells can promote chemotaxis and invasiveness [74–78].

8. CD73 Targeting for Cancer Treatment

Currently, accumulating preclinical data provided by our laboratory and others underscore the therapeutic potential of CD73 blockade for cancer therapy. The analysis of tumorigenesis in CD73-null mice revealed that a lack of CD73 expression can efficiently delay tumors growth and confer metastasis resistance in a variety of murine tumor models [44–46, 72]. All these studies converge on the fact that CD73 KO mice are protected against experimental tumorigenesis because of the absence of Treg-generated adenosine [44, 46]. When CD73 is also expressed on tumor cells, blockade of CD73 on both host and tumor cells is required to achieve optimal antitumor effect [45, 72]. It should be noted that experimental tumorigenesis is also delayed in CD39- or A2A-null mice [79, 80], thus supporting the notion that the adenosinergic system is a relevant target for cancer therapy.

9. Conclusion

CD73 inhibition with α,β-methylene adenosine diphosphate (APCP) or neutralizing monoclonal antibodies (mAb) have demonstrated antitumor effects in various tumor models [73, 81, 82]. Notably, combination of adoptive T-cell therapy with CD73 blockade was shown to be synergistic [45, 81]. This successful combination paves the way for the investigation of other bi- or multitherapies combining CD73 inhibition with immune activating agents (e.g., anti-PD-1, anti-CTLA-4, and anti-TIM-3). CD73 blockade could also be combined with proimmunogenic chemotherapeutic drugs [83, 84]. Apart from their effect on tumor immunity, CD73 antagonists also have the potential to directly inhibit autocrine protumorigenic effects of CD73 on tumor cells, such as increased migration or survival. The use of neutralizing mAbs could be of particular interest as their antagonist properties on CD73 could potentiate their intrinsic immune-mediated cytotoxic activities through the engagement of FcγR on immune cells. Taken together, these observations suggest that CD73 is a promising therapeutic target for the treatment of various cancers. In particular, association of CD73 blockade with other classic or newly developed anticancer agents seems extremely attractive. Future studies aiming at translating these results into therapeutic benefit for patients are warranted.

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Review Article

Ectonucleotidases in Solid Organ and Allogeneic Hematopoietic Cell Transplantation

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

1. Introduction

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

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

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

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

2. Ectonucleotidase Families

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

7. Conclusions

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

Abbreviations

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

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

Conflict of Interests

The authors have no competing financial interests to declare.

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Review Article

The CD39-Adenosinergic Axis in the Pathogenesis of Immune and Nonimmune Diabetes

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Diabetes mellitus encompasses two distinct disease processes: autoimmune Type 1 (T1D) and nonimmune Type 2 (T2D) diabetes. Despite the disparate aetiologies, the disease phenotype of hyperglycemia and the associated complications are similar. In this paper, we discuss the role of the CD39-adenosinergic axis in the pathogenesis of both T1D and T2D, with particular emphasis on the role of CD39 and CD73.

1. Introduction

Extracellular nucleotides, such as adenosine triphosphate (ATP), are important signalling molecules involved in many biological processes. Under basal conditions extracellular concentrations of ATP are maintained at low levels. Endogenous regulation of ATP concentration is mediated by ectoenzymes: the family of ectonucleotidases (E-NTPDases) and ecto-5′-nucelotidase (CD73; E.C. 3.1.3.5) located on the cell surface. Four plasma membrane-bound E-NTPDaess have been cloned: NTPDase1 (CD39; E.C.3.6.1.5), NTPDase2, NTPDase3, and NTPDase8 [1], each with distinct localization and biological properties. NTPDase1 hydrolyzes ATP and adenosine diphosphate (ADP) equally well; NTPDase2 preferentially hydrolyzes ADP; NTPDase3; NTPDase8 have intermediate hydrolysis profiles [2]. The hydrolysis of ATP and ADP generates adenosine monophosphate (AMP), which is then hydrolysed by CD73 to adenosine. CD39 is the rate-limiting enzyme [3] in this cascade and thus is the prime regulator of nucleotide and adenosine concentrations within the microenvironment.

Both CD39 and CD73 expressions are dynamic and change under pathophysiological conditions. Hypoxia upregulates both ectoenzymes—CD39 through Sp1-dependent pathways [4] and CD73 through binding of HIF-1 [5]. Further, within the CD73 gene, promoter region is a cAMP response element (CRE) which regulates transcription through cAMP-dependent CRE-binding protein (CREB). Activation of adenosine receptors increases cAMP and CREB suggesting that the enzymatic product of CD73 (adenosine) may transcriptionally regulate its expression (reviewed in [6]). Finally, the glucocorticoid dexamethasone increases AMP hydrolysis and CD73 expression which is mitigated by protein kinase C (PKC) inhibition [7]. PKC has been shown to activate the transcription of specific genes concluding CD73 [8].

Like ATP, adenosine is constitutively expressed at low levels with a dramatic increase during metabolic stress such as hypoxia and ischemia consequent to ATP hydrolysis. Adenosine is a biologically active molecule that signals through four G-protein-coupled receptors denoted A1, A2A, A2B, and A3. Activation of A1 and A3 inhibits adenylyl cyclase activity through coupling to G_i resulting in a decrease in intracellular cyclic AMP (cAMP), whereas A2A and A2B subtypes are coupled to G_s or G_o to stimulate adenylyl cyclase and lead to an increase of cAMP. A change in cAMP concentrations induces downstream signalling by phosphorylating key enzymes. Furthermore, the A2BR is also coupled to $G_{q/11}$ stimulating phospholipase C (PLC) reviewed in [9] and the A3R signals via PLC-β2/β3 [10]. Adenosine can also activate phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinases (MAPKs) and extracellular receptor

signal-induced kinase (ERK). Additional effector mechanisms include activation of Akt to inhibit apoptosis by A3R, A1R activation which promotes the influx of Ca^{2+} and efflux of K^+ and the activation of the arrestin pathway by adenosine receptors (reviewed in [9]). The adenosine receptors are ubiquitously distributed in the body and the overriding effect of adenosine receptor activation in any one cell is dependent on the repertoire of receptors expressed. The main biological role of adenosine is to maintain vascular and immune homeostasis.

Diabetes, a disorder of glucose homeostasis, is an increasingly prevalent disease worldwide. Two distinct subtypes are recognised: autoimmune diabetes (Type 1 diabetes, T1D) typically afflicting the young and associated with destruction of β-cells and nonimmune diabetes (Type 2 diabetes, T2D) typically arising in those of older age, obese, and with the metabolic syndrome. Although the pathogenesis of the two disorders is distinct, central to both is that of pancreatic β-cell failure and hypoinsulinemia. Features unique to T2D include peripheral insulin resistance and failure of the incretin effect. Despite the disparate aetiologies, the sequelae hyperglycemia and its associated complications are common to both disorders. In this paper, the role of purinergic signalling via the CD39-adenosinergic axis will be discussed in the context of the pathogenesis of T1D and T2D.

2. Pancreatic Expression of Ectoenzymes

Insulin synthesis and secretion is tightly regulated in order to maintain stable blood glucose levels. When blood glucose levels increase, insulin secretion is augmented; conversely in the face of hypoglycaemia, insulin secretion is negligible. Hyperglycemia increases the metabolic demand of β-cells causing a rise in intracellular ATP concentrations [11] and ATP is released with insulin [12] reaching concentrations of 25 μm at the cell surface. Extracellular nucleotides activate P2 receptors—inotropic P2X and metabotropic P2Y. A number of P2 receptors have been implicated in nucleotide-mediated regulation of insulin including P2Y1, P2Y6, P2Y13, P2X3, and amongst others [12–15].

Ectonucleotidase expression has been defined within the mouse, rat, and human endocrine pancreas [16, 17]. Using immunohistochemical and enzyme histochemical techniques, NTPDase1/CD39 was expressed in all blood vessels and acinar tissue; NTPDase2 was localised in capillaries and in connective tissue surrounding islets and acini and NTPDase3 was expressed exclusively in Langerhan islet cells. NTPDase8 was not detected. Inhibition of NTPDase3 activity was shown to facilitate insulin release from rat β-cells. Similarly inhibition of ectonucleotidases with ARL 67156, an inhibitor of NTPDase1 and 3, augmented insulin secretion from human pancreas [13]. Intriguingly CD73 was expressed exclusively in rat islet cells [16] but not in human or mouse [16] (and Chia et al., submitted manuscript). Notably both CD39 and CD73 are secreted from acinar tissue together with ATP directly into the fluid controlling pancreatic exocrine function (reviewed in [18]).

3. Autoimmune Type 1 Diabetes (T1D)

In T1D islet destruction secondary to the autoimmune infiltration of $CD4^+$ T cells and macrophages results in the loss of insulin secretory capacity of β-cells. Treatment with multiple daily injections of insulin slows but does not prevent the development of complications. Transplantation of the whole pancreas or islets is a potential cure for the disease; however, there remains the risk of recurrent disease culminating in graft failure.

3.1. Mouse Models of T-Cell-Mediated Diabetes. The nonobese diabetic (NOD) mouse is the prototypical mouse model for T1D and shares a number of clinical, serological, and immunological features with the human condition. NOD mice spontaneously develop diabetes at ~25 weeks of age after progressing through a prediabetic stage correlating with increasing insulitis.

T-cell-mediated diabetes can also be induced chemically using multiple low dose streptozotocin (MLDS). Streptozotocin is a glucosamine-nitrosourea compound that enters the pancreatic β-cell through the specific glucose transporter 2 (GLUT2) expressed on its surface. Administered in high dose (250 mg/kg) streptozotocin is cytotoxic causing islet death. The onset of diabetes is immediate and there is an absolute lack of insulin. However, streptozotocin administered in low dose (50 mg/kg for 5 days) results in repetitive low-grade β-cell damage, which incites a local inflammatory response comprised principally of $CD4^+$ T cells that is maximal at 12–14 days [19]. The delay in the onset of hyperglycemia suggests immune-mediated damage to β-cells, rather than direct toxicity predominates. Further T cell depleted [20] or deficient mice [21] are resistant to MLDS-induced diabetes.

3.2. Role of E-NTPDase1/CD39 in T-Cell-Mediated Diabetes. $CD4^+$ regulatory T cells are integral to the maintenance of immune homeostasis and abnormalities in number and or function results in autoimmune disease. Indeed low numbers of resting regulatory T cells have been reported in NOD mice [22] and human patients with T1D [23]. CD39 is expressed on both murine [3, 24] and human [25, 26] $CD4^+$ regulatory T cells and is essential for the full suppressive activity of these cells in mice. Further, mice deficient in CD39 (CD39KO) develop an immune diathesis and spontaneous autoimmune alopecia [27]. As anticipated, these mice are highly susceptible to MLDS-induced diabetes with a rapid rate of onset of diabetes (within 10 days) and 100% incidence. Insulitis and reduction in insulin staining was evident at the onset of diabetes. When reconstituted with wild-type bone marrow comprising functional regulatory T cells, the kinetics and incidence are reduced to that of wild-type mice with the development of diabetes at day 42 and overall diabetes incidence of 57% (Chia et al., submitted manuscript). CD39KO mice also have evidence of hepatic insulin resistance [28], which will be discussed in detail below.

Mice have been genetically engineered to overexpress CD39 [29]. CD39 colocalises to β-cells without perturbing

glucose homeostasis [30] and these mice are resistant to MLDS-induced diabetes: minimal insulitis was evident and diabetes occurred in only 14% of animals. This robust protection persisted even following reconstitution with bone marrow from immunodeficient CD39KO mice (Chia et al., submitted manuscript) which may reflect enhanced cell regenerative capacity due to increased pancreatic NTPDase activity. Recent work by Andersson et al. [31] and Annes et al. [32] indicate a role for adenosine signalling in cell specific regeneration. In a zebrafish model, the nonselective agonist NECA did not alter protection against cell death but promoted cell regeneration by increasing the proportion of new cells that proliferate through A2A-dependent mechanisms. Interestingly, NECA did not significantly increase the number of cells in normal development. Further in mice treated with streptozotocin at 150 mg/kg for 2 days, BGL were 30% lower in mice concurrently treated with NECA and cell mass was 8 times larger.

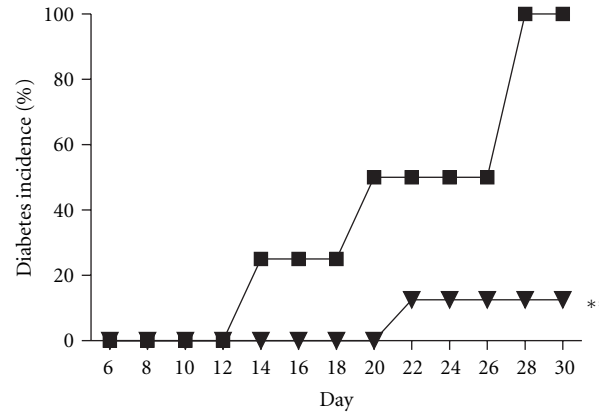

Figure 1: Mice deficient in CD73 are resistant to MLDS-induced diabetes. Diabetes incidence in C57BL/6 wild-type (WT) mice (black squares, $n = 4$) and CD73KO (black triangles, $n = 8$) mice following MLDS. $^{**}P < 0.01$ versus WT mice.

3.3. Role of Ecto-5′-nucelotidase/CD73 in T-Cell-Mediated Diabetes. Although CD73 is not expressed in mouse or human islets [16], it is widely expressed on leukocytes and plays an essential role in leukocyte trafficking. Further, like CD39, CD73 plays an integral role in providing immune competence. CD73 is expressed on $CD4^+$ regulatory T cells in mice [3, 33], but interestingly is not expressed by human $CD4^+$ regulatory T cells [26]. CD73KO mice have been generated [34] and extensively characterised. Markedly, reduced CD73 enzymatic activity [34] results in reduced levels of adenosine [35]. The biological relevance of CD73 had become evident from a number of small animal models: CD73 activity attenuates hypoxia-induced vascular leakage FMLP (formyl-Met-Leu-Phe-OH)-stimulated neutrophil adhesion to endothelial cells and neutrophil accumulation in tissues [34, 36, 37]. CD73KO mice have a proinflammatory phenotype with increased VCAM-1 expression on endothelial cells and heightened susceptibility to vascular inflammation and neointima formation [35]. These effects are a consequence of the loss of both enzymatic and nonenzymatic functions of CD73 [38]. Contrary to these reports, we have shown that CD73KO mice are protected in a model of renal ischemia-reperfusion injury [39–41]. Similarly, CD73KO mice are resistant to MLDS-induced diabetes (Figure 1), presumably a consequence of impaired leukocyte trafficking. In alloxan-induced diabetes in rats, a model which produces a pattern of T1D, both platelet-associated CD39 and CD73 activities are increased [42].

3.4. Adenosine Signalling in T-Cell-Mediated Diabetes. Adenosine signalling has emerged as a regulator of glucose homeostasis through modulating insulin and glucagon release. All four adenosine receptors are expressed in whole pancreas of CD-1 mice [43]; in isolated islets A1, A2A, and A2B receptors are expressed at the mRNA level (Chia et al., submitted manuscript). The A1 and A2A receptors have also been identified on α-cells [44]. Following MLDS, A1 receptor expression is downregulated, A2A expression is unchanged, and A2B receptor expression is augmented (Chia et al., submitted manuscript).

Basal levels of adenosine in isolated islets are in the micromolar range [45], which is sufficient to stimulate glucagon release [46] and inhibit insulin release [47] via the A1 receptor. Thus the peri-islet adenosine concentration is inversely related to extracellular glucose concentrations and may act as a paracrine or autocrine signal [45]. Using the β-cell line INS-1 cells *in vitro*, treatment with the nonspecific agonist NECA or A1, A2A, and A3 agonists reduced insulin secretion in a dose dependent manner. The effect of NECA was completely antagonised by A2B receptor inhibition [48].

In two mouse models of diabetes (cyclophosphamide treated NOD and MLDS), A1 receptor agonism mitigated diabetes but was less efficacious than the nonspecific agonist NECA [43]. In our hands, antagonism or agonism of the A1 receptor did not influence the rate of diabetes in C57BL/6 wild-type (WT) mice (Chia et al., submitted manuscript).

The A2A receptor is widely expressed on both tissues and circulating cells. Mice lacking the A2A receptor (A2ARKO) are highly susceptible to MLDS-induced diabetes with rapid onset (within 10 days) and 100% diabetes incidence. Like CD39KO mice, the A2ARKO mice are immunocompromised and have hyperproliferative T cells [3]. To delineate the site-specific importance of the A2A receptor, a series of adoptive transfer experiments were performed. Deletion of the A2A receptor either on the tissues or the circulating cells increased the susceptibility of these mice to the effects of MLDS (Chia et al., submitted manuscript). NECA ameliorated diabetes in A2ARKO mice and treatment with an A2AR agonist had no effect in wild-type mice following MLDS [43].

The prevention of MLDS-induced diabetes in CD-1 mice by NECA was reversed by pretreatment with a selective A2B receptor inhibitor [43]. Similarly, we have identified a role for the A2B receptor particularly in the early response to MLDS. The rise in blood glucose following MLDS in C57BL/6 wild-type mice was quicker, reaching hyperglycemia by 8–10 days,

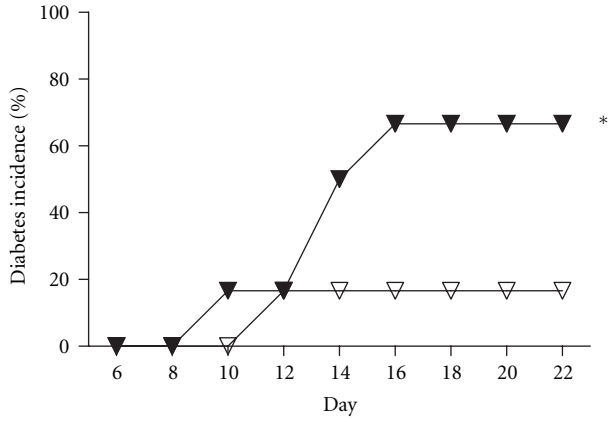

Figure 2: Inhibition of A2B receptor in CD73KO mice increases susceptibility to MLDS-induced diabetes. Diabetes incidence of CD73KO mice treated with either saline (open triangles, $n = 6$) or the A2BR inhibitor (dose: 0.5 μg/g body weight (BW), twice daily) (black triangles, $n = 6$). $^*P < 0.05$ versus saline-treated mice.

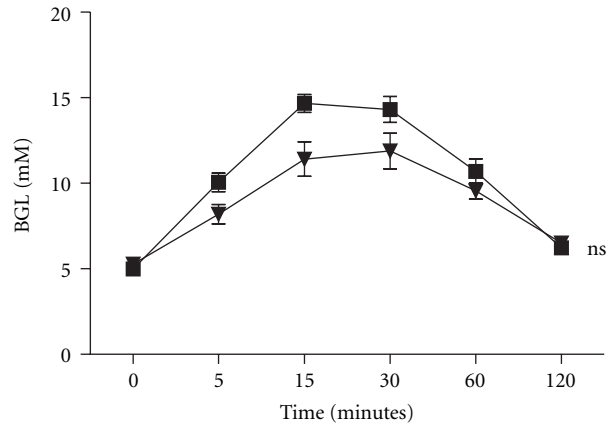

Figure 3: Normal glucose handling in CD73KO mice. Blood glucose levels following 1 mg/g BW of intraperitoneal glucose. WT mice (black squares, $n = 6$); CD73KO mice (black triangles, $n = 8$); ns—not significant versus WT mice.

although the overall rate of diabetes was unchanged (Chia et al., submitted manuscript).

The protection conferred by CD39 overexpression was mitigated by deletion of the A2A receptor or by pharmacological inhibition of the A2B receptor. Complete blockade of both receptors did not further exaggerate the diabetic phenotype (Chia et al., submitted manuscript). Involvement of more than one adenosine receptor parallels the effects of adenosine in renal IRI, where A2A receptor signaling predominates on circulating $CD4^+$ T cells [49] and macrophages [50], while A2B receptor signaling within the renal parenchyma is also important [51]. Intriguingly, CD73KO mice coadministered with an A2B receptor inhibitor became susceptible to the effects of MLDS, with an onset of diabetes at day 10 and a diabetes incidence of 66% (Figure 2).

4. Nonimmune Type 2 Diabetes (T2D)

Insulin resistance characterises T2D, however, β-cell dysfunction must coexist for hyperglycemia to occur. Indeed it is progressive β-cell dysfunction that underpins the progression from normoglycemia to impaired glucose tolerance to overt diabetes.

4.1. Role of CD39 and CD73 in T2D. Mice deficient in CD39 demonstrate impaired glucose tolerance following oral glucose tolerance testing a consequence of hepatic insulin resistance rather than peripheral muscle resistance. There was an associated increased level of hepatocyte c-Jun NH_2-terminal kinase (c-JNK) in response to extracellular nucleotides and aberrant insulin receptor substrate (IRS)—2 phosphorylation in the liver of these mice [28]. There was no abnormality in glucose handling following an intraperitoneal glucose load in mice overexpressing CD39 [30] nor intriguingly in CD73KO mice (Figure 3). In human T2D, CD39 expression has been determined in peripheral blood mononuclear cells. Poor glycemic control was associate with proportions of CD39+ cells particularly within the CD19+ subset. Further an increase in CD39 enzymatic activity was observed in this patient cohort [52]. Further, platelet-associated CD39 enzymatic activity was increased in patients with T2D, hypertension, and coexisting T2D and hypertension. Platelet-associated CD73 enzymatic activity was only increased in patients with hypertension or coexisting hypertension and T2D and not T2D alone [53]. CD39 expression also influences the susceptibility to diabetes-induced renal disease in both mice [54] and humans [55]. In African Americans, a common ENTPD1 (CD39) two-single nucleotide polymorphism haplotype was associated with an increased risk for end stage renal disease secondary to T2D.

4.2. Adenosine Signalling in T2D. All adenosine receptors are expressed at the mRNA level in skeletal muscle of mice [56] and the role of adenosine receptor blockade in reversing insulin resistance in skeletal muscle from diabetic rats has been realised for some time [57, 58]. In keeping with this treatment of wild-type C57BL/6 mice with NECA promoted impaired glucose tolerance by inhibiting glucose disposal [59]. Although initially thought to be mediated by the A1 receptor, studies with A1RKO [56] and A2RKO [59] mice show that these receptors have a minimal effect on skeletal muscle uptake of glucose. Rather it appears that activation of A2B receptor promotes peripheral insulin resistance and blockade of the receptor in diabetic KKA^Y mice enhances glucose disposal into skeletal muscle and adipose tissue as well as reducing hepatic glucose production [59]. Further, in Goto-Kakizaki rats, which resemble T2D, insulin levels were increased temporarily following A2B receptor inhibition, although without effecting blood glucose level [48]. There may however be a role for A1 receptor activation through the suppression of lipolysis and free fatty acid levels (FFA) [60] both of which are involved in the pathogenesis of

T2D. Indeed, mice overexpressing the A1 receptor in diet-induced insulin resistant mice have lower FFA levels and insulin resistance compared to controls [61]. The effect of the null mutation of A1R on glucose homeostasis following a high fat diet is controversial: Faulhaber-Walter et al. [62] demonstrated decreased glucose tolerance with increased BGL and insulin levels in A1RKO mice (C57BL/6 and Swiss compared to controls) as early as 5 weeks following a high fat diet. Yang et al. [63], however, reported A1RKO mice (C57BL/6) clear blood glucose more efficiently, however, following a high fat diet both WT and A1RKO mice develop glucose intolerance.

4.3. Adenosine and the Incretin Effect. The incretin hormones glucagon-like peptides-1 (GLP-1) and glucagon intestinal peptide (GIP) are released from the gastrointestinal tract in response to food and promote insulin secretion in a glucose concentration-dependent manner in β-cells and inhibit glucagon secretion. The incretins are rapidly metabolised by dipeptidyl peptidase-4 (DPP-4) and drugs that inhibit this enzyme are very effective in the treatment of T2D. DDP-4, also known as CD26 or adenosine deaminase (ADA), enzymatically and irreversibly converts adenosine to inosine. ADA activity has been found in most organs but is notably high in adipose tissue, liver, skeletal muscle and heart. An increase in ADA activity has been reported in patients with T2D and a relationship with insulin resistance has been postulated [64]. High ADA activity is associated with low adenosine levels; however a direct relationship between adenosine and the incretin effect in T2D has not yet been defined.

5. Concluding Remarks

The CD39-adenosinergic axis is involved in the pathophysiology of pancreatic dysfunction and thus drug development targeting different components of the pathway may be of relevance in the treatment of both type 1 and type 2 diabetes. There remain a number of unanswered questions including the source of CD73 enzymatic activity given the lack of expression within the pancreas; the mechanisms behind protection observed with CD73 deletion in MLDS- induced diabetes and the role of purinergic signalling in the incretin effect, which is of particular importance in the pathogenesis of T2D.

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Research Article

Production of Adenosine by Ectonucleotidases: A Key Factor in Tumor Immunoescape

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It is now well known that tumor immunosurveillance contributes to the control of cancer growth. Many mechanisms can be used by cancer cells to avoid the antitumor immune response. One such mechanism relies on the capacity of cancer cells or more generally of the tumor microenvironment to generate adenosine, a major molecule involved in antitumor T cell response suppression. Adenosine is generated by the dephosphorylation of extracellular ATP released by dying tumor cells. The conversion of ATP into adenosine is mediated by ectonucleotidase molecules, namely, CD73 and CD39. These molecules are frequently expressed in the tumor bed by a wide range of cells including tumor cells, regulatory T cells, Th17 cells, myeloid cells, and stromal cells. Recent evidence suggests that targeting adenosine by inhibiting ectonucleotidases may restore the resident antitumor immune response or enhance the efficacy of antitumor therapies. This paper will underline the impact of adenosine and ectonucleotidases on the antitumor response.

1. Introduction

Tumor immunology is an intensely investigated field of research, even though its clinical applications in the field of cancer treatment are currently limited. It is now well established that the molecular mechanisms leading to cell transformation and cancer generation induce the appearance of neoantigens and danger signals. These molecules give rise to the immune response which drives tumor rejection (a phenomenon called immunosurveillance), but some cancer cells escape this rejection by limiting tumor antigen expression (a phenomenon called immunoediting) mainly by inducing active immune tolerance mechanisms [1]. These mechanisms include the proliferation and local accumulation of immunosuppressive cells, including regulatory T cells (Tregs), Th17 cells, and myeloid-derived immunosuppressive cells (MDSCs). This tolerance (a phenomenon called immunoescape) prevents cancer rejection by the immune system and blunts the efficacy of immunotherapy [2]. All these events have been clearly demonstrated in mice models for years.

In humans, recent data demonstrate that infiltration of the tumor bed by CD8 and memory T cells correlates with good outcomes, while tumor-bed infiltration by immunosuppressive cells correlates with poor outcomes [3–5]. Such data raise the hypothesis that the immune response also controls tumor growth in humans. We may wonder whether therapies that shift immune tolerance towards the antitumor immune response could lead to tumor eradication. Chemotherapies such as cyclophosphamide, 5-Fluoro ruracil, and gemcitabine [6–9] by their capacity to eliminate immunosuppressive cells such as Tregs and MDSCs can restore the antitumor immune response. On the other hand, it is now widely accepted that the antitumor efficacy of many chemotherapy drugs is in part due to their induction of antitumor immune responses [10–12]. In addition, drugs, like anti-CTLA-4 mAb and anti-PD1 mAb, that directly target immune suppression, have either been approved by FDA or are under clinical investigation in many cancer types with very impressive clinical results [13].

Many strategies are currently used to target immune suppression. One is to target adenosine (a purine nucleoside)

or enzymes that catalyze the generation of adenosine, namely, ectonucleotidase molecules CD39 and CD73. In this paper, we will propose a synthetic focus on the impact of this pathway on the antitumor immune response and its therapeutic potential. For this, we will describe not only the effect of adenosine on cancer cells, immune cells, and endothelial cells, but also how adenosine is produced by ectonucleotidase expressing cells.

2. Effect of Adenosine in the Context of Cancer

Adenosine is constitutively present in the extracellular media at a very low concentration, but its concentration increases in many metabolically stressful conditions, notably in the tumor microenvironment [14, 15]. Following its release, adenosine binds to membranous adenosine receptors, which belong to a family of G-protein-coupled receptors [16]. This family is composed of four different members called adenosine A1, A2A, A2B, and A3 receptors, which mediate different cellular pathways through adenosine binding. A1 and A3 receptors induce a decrease in intracellular cAMP, while A2A and A2B receptors induce activation of adenylate cyclase resulting in increased intracellular levels of cAMP. A1 and A3 receptors also induce the activation of phosphatidyl-Inositol 3 kinase (PI3K) and protein kinase C (PKC). At low concentrations of adenosine, only high-affinity A1, A2A, and A3 receptors are involved, whereas at high concentrations, like those observed in the tumor microenvironment, the low-affinity A2B receptor is involved in the signaling [17].

Because adenosine receptors are widely expressed, adenosine can influence immune, cancer, and endothelial cell functions (Figure 1).

2.1. Adenosine and Its Effect on the Immune System. Taking into account the different affinities between adenosine and its receptors and the fact that adenosine receptors are differentially expressed depending on the cell type, adenosine has the ability to act variably on immune cells. Adenosine binding to A1 or A2B receptors on neutrophils thus induces their activation, promotes their inflammatory activity, and induces chemotaxis and adherence of neutrophils to endothelial cells. In the context of cancer, neutrophil activation may be deleterious notably because neutrophils are able to produce metalloproteases, which foster matrix modification and promote metastases. Neutrophils can also promote chronic inflammation, which promotes tumor growth [18, 19].

Macrophages are also affected by adenosine. A2A receptor activation switches macrophages from an M1- to an M2-like phenotype. This switch needs the previous activation of macrophages by TLR (toll-like receptor) agonists to upregulate the A2A receptor. This event enhances the capacity of macrophages to produce VEGF (vascular endothelial growth factor) and IL-10, two cytokines that promote tumor growth [20].

Adenosine has been shown to promote MDSC functions in an A2B receptor-dependent manner. Indeed, adenosine leads to MDSC expansion and may promote tumor tolerance in this way [21].

Adenosine could act on the A2A receptor of natural killer (NK) cells and could blunt their capacity to produce tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) [22]. In addition, increased levels of adenosine in the tumor microenvironment inhibit the lytic activity of NK cells in an A2A receptor-dependent manner [23]. Adenosine also inhibits both perforin and FasL cytotoxic molecules, thus, limiting the ability of NK cells to mediate the lysis of tumor cells. Adenosine could also modify NKT cell response by increasing their production of IL-4 and transforming growth factor-β (TGF-β), while decreasing their production of IFN-γ [24].

Dendritic cells (DCs) are a critical component of the immune response and are aimed at controlling T-cell polarization. During tumor growth, DCs invade the tumor bed and differentiate under hypoxic and inflamed conditions. In this context, DCs are in contact with high concentrations of adenosine. The stimulation of adenosine receptors skews DC differentiation towards a distinct cell population characterized by the expression of both DC and macrophage cell surface markers. Pharmacologic analysis identified the A2B receptor as the mediator of adenosine's effects on DCs. Unlike normal myeloid DCs, adenosine-differentiated DCs have impaired allostimulatory activity and express high levels of angiogenic, proinflammatory, immune suppressor, and tolerogenic factors, including VEGF, IL-8, IL-6, IL-10, Cyclo-Oxygenase-2 (COX-2), TGF-β, and Indoleamine 2,3-dioxygenase (IDO) [25]. In addition, they promote tumor growth in mice. However, the overall effect of A2B activation on DCs is not fully understood because some other reports suggest that adenosine could increase IL-6 production and favor Th17 responses [26].

Adenosine could also have a major impact directly on T cell subsets. Signaling through the TCR (T cell receptor) causes a rapid increase in A2A receptor mRNA levels, which correlate with a significant increase in cAMP accumulation in these cells [27]. *In vitro*, antigen recognition in the setting of A2A receptor activation by specific agonists induces T-cell anergy, even in the presence of costimulation such as CD28 triggering [28]. T cells initially stimulated in the presence of an A2A receptor agonist also fail to proliferate and to produce IL-2 and IFN-γ after restimulation. Engagement of an A2A receptor *in vivo* inhibits IL-6 expression while enhancing the production of TGF-β. TGF-β in the absence of IL-6 promotes the differentiation of naive T cells into Treg cells. Consequently, treating mice with adenosine agonists not only inhibits Th1 effector cell generation but also promotes the generation of Tregs [28]. In conclusion, exposure to adenosine during T cell activation promotes long-term T-cell anergy and the induction of Tregs, both of which lead to a drastically impaired antitumor immune response.

2.2. Effects of Adenosine on Cancer Cells. Adenosine may affect cancer growth through direct binding on its specific receptors expressed at the cell surface of tumor cells (Figure 2). More particularly, A1 receptor is mainly involved in tumor cell proliferation and induces activation of the cell cycle. A1 receptor could inhibit p27, a molecule that promotes senescence and limits proliferation [29]. A3 receptor

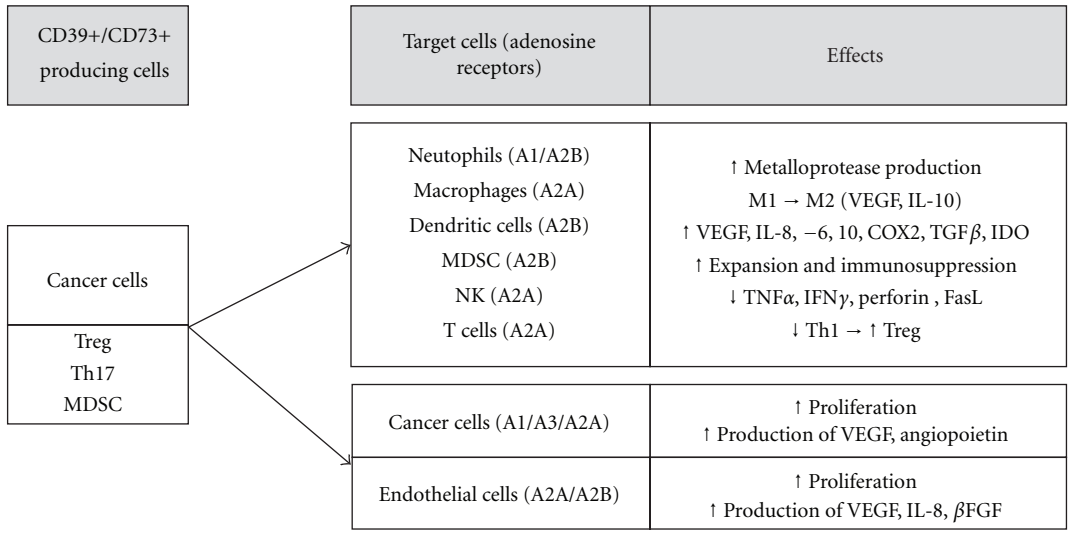

Figure 1: Effects of adenosine produced by CD39+/CD73+ cells on target cells. Cancer cells, Tregs, Th17, and MDSCs could produce adenosine through degradation of ATP/ADP by CD39 and CD73. Then adenosine binds on target cells, such as immune cells, cancer cells, or endothelial cells and modifies their activity.

is expressed in many cancers and seems to be overexpressed in cancer cells compared with normal cells [30]. The major effect of A3 receptor activation is to promote angiogenesis. Adenosine, in an A3-dependent manner, increases hypoxia-inducible factor-1α (HIF-1α) protein expression in response to hypoxia in human melanoma, glioblastoma, and colon cancer cells [31–33]. Adenosine also mediates the production of VEGF and Angiopoietin by tumor cells in an A3-dependent manner [34]. Some reports have also demonstrated that human chronic lymphocytic leukemia (CLL) [35], myeloma [36] and melanoma cells [37] express functional A2A receptors. Activation of these receptors could modulate the response to chemotherapy. The A2A receptor also increases erythropoietin (EPO) production in hepatocellular carcinoma (Hep3B) cells [38].

Adenosine does not always induce cancer cell proliferation; some reports mentioned that adenosine could also induce cancer cell death or inhibit cell proliferation [17].

2.3. Effects of Adenosine on Endothelial Cells. A2A and A2B receptors exert a strong proangiogenic effect. The A2A receptor is expressed by endothelial cells and is associated with vasodilation [39]. A2A mediates the production of VEGF and the proliferation of endothelial cells [40]. A2B receptors are expressed in human neoangiogenic endothelial cells, where they play a role in the regulation of the expression of angiogenic factors like VEGF, interleukin-8 (IL-8), and βFGF (basic fibroblast growth factor) [41, 42]. A2B receptors are also involved in mRNA and protein increases of IL-6 in human astrocytoma cells, thus, promoting STAT3 (Signal transducer and activator of transcription 3) mediated angiogenesis [43].

3. Production of Adenosine by Ectonucleotidases

The dominant pathway leading to extracellular adenosine production is the extracellular dephosphorylation of ATP by ectonucleotidases. This degradation requires two enzymes called CD39 (*ectonucleoside triphosphate diphosphohydrolase 1*-Entpd1) and CD73 (*ecto*-5′-*nucleotidase*-Nt5e). CD39 hydrolyzes ATP and ADP to AMP, which is further hydrolyzed to adenosine by CD73. The conversion of ATP into AMP by CD39 is reversible by the action of extracellular kinases such as adenylate kinase. By contrast, the conversion of AMP into adenosine by CD73 is reversible only after the transport of adenosine into cells where it can be converted into AMP by adenosine kinase. CD39 is expressed on endothelial cells [44] and on many types of activated hematopoietic cells such as B cells, NK cells, and activated T cell subsets and also on monocytes/macrophages and dendritic cells [45, 46]. CD39 degrades ATP produced by activated platelets and thus inhibits the generation of thrombi, and may act on tumor angiogenesis by this pathway [47]. CD39 expression on leukocytes is indispensable for the generation of adenosine and consequently dictates their immunosuppressive functions [48].

CD73 is considered the rate-limiting enzyme in the generation of extracellular adenosine [49]. CD73 catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleosides. This molecule notably drives the conversion of AMP into adenosine. This antigen is expressed on some immune cells such as activated B but not on naive T cells [50, 51], endothelial cells [44], follicular dendritic cells [52], epithelial cells [53], and fibroblasts [54]. In the tumor microenvironment, CD73 expression is regulated by hypoxia [55–57]. In addition, some factors found in the tumor

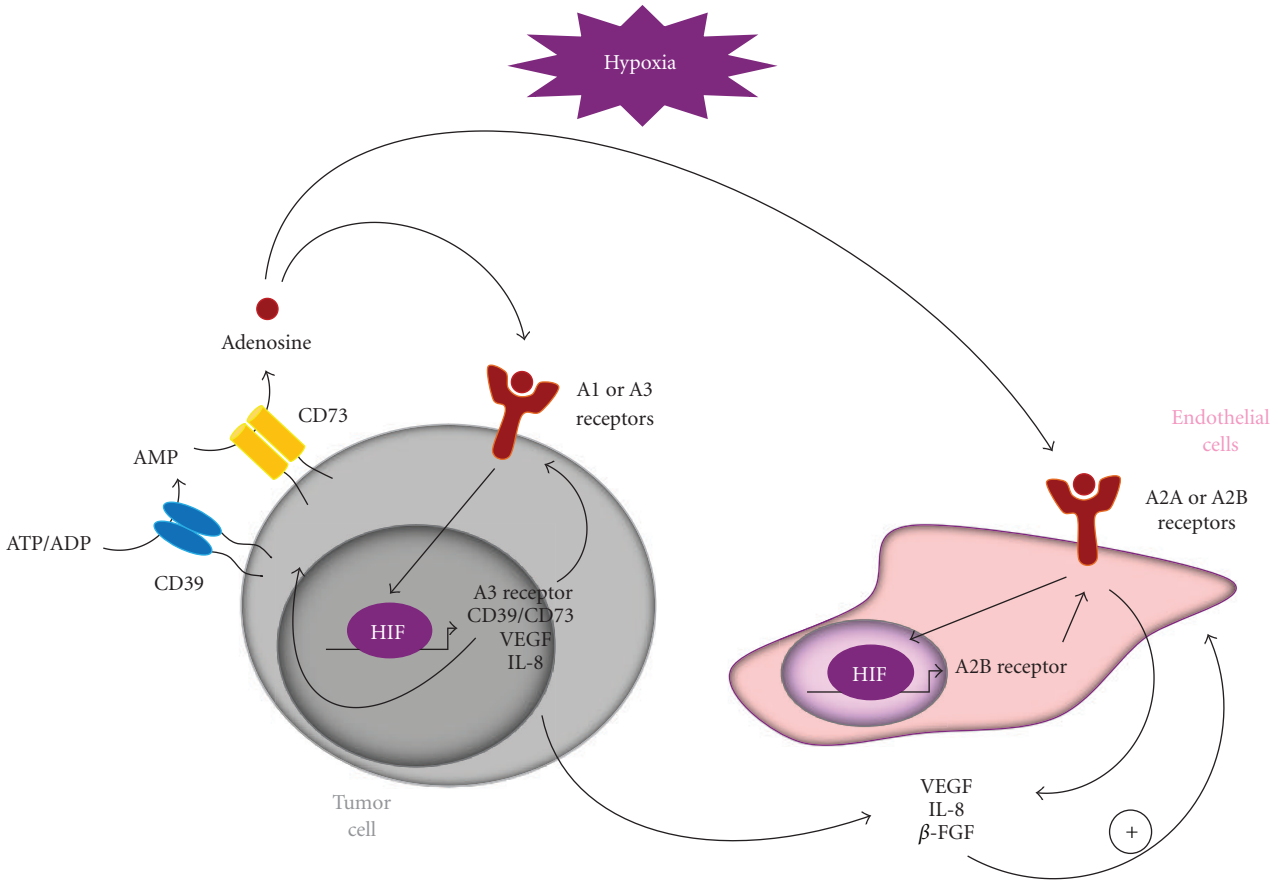

Figure 2: Hypoxia mediated expression of ectonucleotidases and adenosine receptors. HIF is induced under hypoxic conditions in cancer cells and directly increases the expression of (1) ectonucleotidases CD39 and CD73, which generate adenosine from ATP/ADP, (2) adenosine receptors that could, after binding of adenosine, activate HIF, and (3) angiogenic molecules VEGF and IL-8. These (again with β-FGF) also produced by endothelial cells (through binding of adenosine on specific receptors) could induce proliferation of these cells.

microenvironment such as TNF-α, IL-1β [58], prostaglandin E2 (PGE2) [59], TGF-β [60], and IL-6 [61] could also upregulate ectonucleotidase expression, whereas IFN-γ and IL-4 have been shown to downregulate CD73 expression [59, 62].

4. Expression and Role of Ectonucleotidases in Cancer

Many studies on CD39 or CD73-deficient animals have shown that the expression of ectonucleotidase on cancer cells and on host cells (hematopoietic and nonhematopoietic cells) is involved in tumor progression [63–68].

4.1. Effects and Expression of Ectonucleotidases on Cancer Cells. Ectonucleotidase expression has been observed in many human cancer types such as melanoma, breast cancer, colon cancer, glioma, leukemia, gastric, and head and neck cancers [69]. The prognostic role of ectonucleotidase expression in cancer cells remains largely unclear. In a small cohort of breast cancer patients, expression of CD73 in cancer cells seemed to correlate with survival [70]. In a cohort of colorectal cancer patients, high expression of CD73 was observed in about 50% of tumor samples and this overexpression correlated significantly with poor tumor differentiation, nodal status, and a high T stage. Overall survival in patients with high expression of CD73 was poorer than in patients with low expression of CD73 [71]. The expression of ectonucleotidases on chronic lymphocytic leukemia cells correlates with a better prognosis [72]. In another study, CD39 was found to be widely expressed in CLL lymph nodes, whereas CD73 is only expressed in proliferative centers. Ectonucleotidase-expressing LLC cells produce adenosine, which mediates drug-induced resistance via an AMPc-dependent autocrine loop [35].

For example, immunoediting may select cancer cells that highly express ectonucleotidases; that is, cells better armed to fight against the antitumor immune response. HIF induced under hypoxia could increase ectonucleotidase expression through direct binding on response elements located within the ectonucleotidase promoters [55].

Ectonucleotidase expression on tumor cells and on tumor exosomes (small vesicles secreted by cancer cells) may increase local concentrations of adenosine and could blunt the antitumoral immune response [73, 74]. Indeed, blockade of the A2A receptor on CD8 T cells inhibits the growth of strongly immunogenic melanomas [73]. A2B receptor blockade acts on DC subsets and enhances tumor Ag presentation and cytokine-mediated T cell activation [75].

Adenosine-receptor promoters contain an HIF-1α response element that drives expression of these receptors in hypoxic cells including endothelial cells [76], cancer cells [77], and DCs [78]. In fact, adenosine receptors have been found in many cancer types in mice and humans. Thus hypoxia induces a vicious circle involving the adenosine pathway, by enhancing the production of adenosine via the upregulation of both ectonucleotidases and adenosine receptors.

4.2. Ectonucleotidase Expression on Tregs. Tregs are one of the key immunosuppressive cells in the context of cancer. Regulatory T cells (Tregs) were initially identified in both mice and humans as CD4+ T cells constitutively expressing CD25 and inhibiting the immune response of effector T cells. In cancer-bearing animals or patients, Tregs expand, migrate to tumor sites, and suppress the antitumor immune response mediated by NK cells, $CD4^+$, $CD8^+$ T cells, and myeloid cells, through different molecular mechanisms [79]. In experimental tumor models as well as in cancer-bearing patients, the accumulation of Tregs generally progresses during tumor growth. Treg accumulation was first described among peripheral blood leukocytes in cancer-bearing patients [80], in the spleen of tumor-bearing rodents and also in the tumor itself [81], where a high infiltration of Tregs correlates with a poor prognosis in most cancer types [82]. Therefore, Tregs are usually considered a major cell population involved in immune tolerance, which protects cancer cells from antitumor immunity.

The mechanism of Treg-mediated immunosuppression remains unclear, and many mechanisms of action have been proposed. Recently, murine Tregs were shown to express membranous CD73 and CD39 and to be able to transform ATP into adenosine. Functionally, the coexpression of CD39 and CD73 with the pericellular generation of adenosine dictates the suppressive functions of Treg cells on A2A-positive effector T cells [83]. In humans, ectonucleotidases have also been observed on Tregs [84]. Tregs could be induced from naive T cells by TCR triggering in the presence of TGF-β. A recent report demonstrated that triggering of the TCR induced expression of CD39 and CD73 on these cells [60]. Finally CD39 expression on Tregs has also been shown to inhibit NK cell activity and to promote hepatic metastasis in a murine melanoma cancer model [64] and T cell anergy in human follicular lymphoma [85]. However, the molecular mechanism that leads to ectonucleotidase expression in Tregs and their role in the control of tumor growth remains unclear.

4.3. Ectonucleotidase Expression on Th17 Cells. Th17 cells are $CD4^+$ T cells developed by TCR triggering with a combination of IL-6 and TGF-β. After induction, IL-23, an IL-12 family member, maintains Th17 cell polarization [86]. Th17 cells have emerged as key participants in a wide range of autoimmune disorders, including inflammatory bowel disease, psoriasis, and ankylosing spondylitis [87]. Th17 expansion has been shown in the blood, bone marrow, and spleen of tumor-bearing mice. Th17 cell expansion has also been observed in human cancers such as melanoma, prostate cancer, fibrosarcoma, and advanced head and neck cancers [88–90], and Th17 infiltration is associated with a poor outcome in colon and liver cancers [91, 92]. The role of Th17 cells in cancer immunity remains controversial. Many reports have suggested that Th17 may promote tumor growth in mice and humans. IL-17 produced by Th17 has been shown to promote angiogenesis and inflammation through STAT3 signaling and MDSC mobilization [93, 94]. On the other hand, the adoptive transfer of tumor-specific Th17 cells could control tumor growth as a result of their ability to promote cytotoxic T cell activation [95, 96]. These studies suggest that Th17 cells may exert regulatory or inflammatory functions in the context of cancer depending on the cytokine microenvironment. Our recent work tried to reconcile these discrepancies. We made the seminal observation that *in vitro* Th17 cells generated with IL-6 and TGF-β express CD39 and CD73, while inflammatory Th17 cells generated with IL-6, IL-1β, and IL-23 do not. Ectonucleotidase expression on Th17 leads to adenosine release and the suppression of effector T cells. The expression of ectonucleotidases was dependent on IL-6-driven STAT3 activation and TGF-β-mediated downregulation of zinc finger protein growth factor independent-1 (Gfi-1), both of which transcriptionally regulate ectonucleotidase expression during Th17 cell differentiation. Ectonucleotidase expression on Th17 cells is relevant in the context of tumor growth as wild-type Th17 cells promote tumor growth while Th17 cells obtained from CD39-deficient mice remain unable to affect tumor growth. Thus, our data suggest that the expression of ectonucleotidases dictates the immunosuppressive fate of Th17 cells in cancer [61].

4.4. Ectonucleotidase Expression on MDSCs. Myeloid-derived suppressor cells (MDSCs) have been identified in cancer patients and in tumor-bearing mice as a population of immature myeloid cells with the ability to suppress T cell activation. In mice, MDSCs are uniformly characterized by the expression of the cell-surface antigens Gr1, Ly-6C/G, and CD11b, while in humans, MDSCs are typically found in the $Lin^-CD11b^+CD33^+HLA\text{-}DR^-$ subset. Given that MDSCs from naive mice were generally found to lack immunosuppressive properties, it has been shown that MDSCs require activation signals, such as cytokines or exosome membrane-bound Hsp72, from tumor cells to support their suppressive functions on T cells [97, 98].

Recently, Ryzhov et al. have shown that $CD11b^+Gr1^{high}$ $Ly\text{-}6G^+$ cells express high levels of CD73 at the cell surface. This correlates with high *ecto-5′-nucleotidase* enzymatic activity, which contributes to the expansion and the immunosuppressive properties of MDSCs [21]. The relevance of these observations in the control of tumor progression needs to be established.

5. Inhibition of Ectonucleotidase Activity as a Therapeutic Approach in Cancer

On the assumption that adenosine production promotes tumor proliferation, neoangiogenesis, and directly blunts antitumor effector cells, and that ectonucleotidases are highly

expressed on tumor cells (and correlate with a poorer overall survival rate) and on immunosuppressive cells, it should be of great interest to inhibit the adenosine receptor on target cells or adenosine production by ectonucleotidases to promote the antitumor response.

Even though targeting adenosine receptors seems to be relevant, it could have an uncertain impact on tumor treatment. For example, it has been shown that targeting A2A receptors could dampen etoposide-mediated CLL cell death [35], while enhancing the effects of melphalan, lenalidomide, bortezomib, and doxorubicin on multiple myeloma [36].

Thus, targeting ectonucleotidase activity seems to be more appropriate. Inhibition of CD39 activity by polyoxometalate1 (POM-1), a pharmacologic inhibitor of nucleoside triphosphate diphosphohydrolase activity, abrogated melanoma tumor growth in wild-type mice but not in CD39-null animals indicating a specific effect of POM-1 on host CD39 [64].

The inhibition of CD73 has been more thoroughly studied. A specific blocking antibody suppressed the growth of established 3-methylcholanthrene-induced tumors or prostate tumors and inhibited the development of lung metastases [99]. Moreover the inhibitor α,β-methyleneadenosine 5′-diphosphate (APCP) also affected thymoma or ovarian tumor growth and B16F10 lung metastasis formation [66–68].

Recent reports demonstrate that chemotherapies, in addition to their cytotoxic effects, mediate an immune effect via the release of ATP, emphasizing the importance of inhibiting ectonucleotidases [10, 100]. The use of ectonucleotidase inhibitors or blocking antibodies in association with chemotherapies that facilitate ATP production could thus be focused on patients with ectonucleotidase-overexpressing tumors. For this, patients should be screened for ectonucleotidase expression on cancer cells and on tumor infiltrating cells, and ATP producing chemotherapeutic drugs should be selected, notably thanks to the *in vivo* imaging method that allows the real-time measurement of ATP within the tumor interstitium developed by the Di Virgilio team [101].

6. Conclusion

Clear understanding of the mechanisms involved in tumor-induced tolerance is a capital objective to develop effective antitumor immunotherapies. It is clear that ectonucleotidase expression on cancer cells as well as immune cells that infiltrate the tumor bed facilitates tumor development. Adenosine production that results from the transformation of extracellular ATP by ectonucleotidases promotes tumor cell proliferation, neoangiogenesis and blunts antitumor effector cells. Thus a promising strategy to simultaneously reduce these effects would be to target ectonucleotidases using blocking antibodies or inhibitors of ectonucleotidases or adenosine receptors. This inhibition could be a new avenue to explore to improve the efficacy of classical cytotoxic agents by enhancing extracellular ATP levels which would sustain the antitumor immune response.

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Research Article

CD73 Is Critical for the Resolution of Murine Colonic Inflammation

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CD73 is a glycosyl-phosphatidylinositol-(GPI-) linked membrane protein that catalyzes the extracellular dephosphorylation of adenosine monophosphate (AMP) to adenosine. Adenosine is a negative regulator of inflammation and prevents excessive cellular damage. We investigated the role of extracellular adenosine in the intestinal mucosa during the development of Dextran-Sulfate-Sodium-(DSS-)salt-induced colitis in mice that lack CD73 ($CD73^{-/-}$) and are unable to synthesize extracellular adenosine. We have found that, compared to wild-type (WT) mice, $CD73^{-/-}$ mice are highly susceptible to DSS-induced colitis. $CD73^{-/-}$ mice exhibit pronounced weight loss, slower weight recovery, an increase in gut permeability, a decrease in expression of tight junctional adhesion molecules, as well as unresolved inflammation following the removal of DSS. Moreover, colonic epithelia in $CD73^{-/-}$ mice exhibited increased TLR9 expression, high levels of IL-1β and TNF-α, and constitutive activation of NF-κB. We conclude that CD73 expression in the colon is critical for regulating the magnitude and the resolution of colonic immune responses.

1. Introduction

Many mechanisms are proposed to lead to chronic inflammation of the gut mucosa, which is exemplified by Crohn's disease (CD) and ulcerative colitis (UC), also known as inflammatory bowel diseases (IBDs) [1]. These include dysregulation of the innate immune response to enteric flora [2, 3], increased epithelial barrier permeability [4], and defective regulation of the adaptive immune response [5]. Several susceptibility genes carrying mutations have been found in patients suffering from IBD [1]. Mutations in the human leukocyte antigen genes are associated with both CD and UC [6], while mutations in the Nuclear Oligomerization Domain (NOD) 1/2 genes are associated with CD [1, 7].

The passage of soluble molecules across the epithelial barrier of the gastrointestinal tract is a tightly regulated process [8]. Nutrients must be absorbed into the bloodstream, while commensal bacteria normally found in the GI tract, and their associated antigens, must be kept isolated from intraepithelial lymphocytes to avoid abnormal and persistent immune responses that can cause inflammation or other types of trauma [8, 9]. The purine nucleoside adenosine, its receptors and ectoenzymes (CD39 and CD73) are emerging as critical players in the regulation of intestinal immune-mediated inflammation [10–12].

During inflammation and the initiation of primary immune responses, adenosine triphosphate (ATP) is released into the extracellular environment following cell damage [13]. Extracellular ATP, a damage-associated molecule and a potent activator of the immune response, increases the production of proinflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-12. ATP also enhances the potency of the oxidative stress response of activated macrophages [13]. The immune system has various mechanisms to resolve these inflammatory signals. One such mechanism is activated by the extracellular adenosine generated through the activities

of CD39 and CD73 [14]. CD39 catabolizes extracellular ATP to adenosine monophosphate (AMP) while CD73 (a 5′-ectonucleotidase) further converts AMP to adenosine [15]. Adenosine inhibits production of TNF-α, IL-1β, IL-6, IL-12, and IL-23 by monocytes and dendritic cells (DCs) and promotes the production of IL-10 [16]. Therefore, CD73-generated adenosine acts as a negative feedback signal to prevent uncontrolled inflammation that would otherwise cause collateral damage to healthy tissues.

CD39, CD73, and adenosine receptors are expressed on intestinal epithelial cells and various immune cell types [17–19]. CD39 and CD73 have recently been shown to be important for T regulatory cell function [20], and mice lacking these enzymes exhibit very severe IBD in trinitrobenzene sulfonate (TNBS) colitis model [10, 11]. Adenosine mediates its effects through four G protein-coupled receptors: A_1AR; A_{2A}AR; A_{2B}AR; A_3AR [14]. Adenosine receptors are expressed in the intestine of mice and humans, and signaling through these receptors is critical for regulation of the severity of intestinal inflammation following a wide range of insults [16]. For example, activation of the A_{2A} receptor has been shown to reduce intestinal inflammation in mice by decreasing leukocyte infiltration and inhibiting proinflammatory cytokines [21], while inhibition of adenosine kinase improves IBD pathology in DSS-induced colitis [22].

Because of the anti-inflammatory role of adenosine and its association with IBD, we investigated the role of extracellular (CD73-generated) adenosine in DSS-induced colitis in mice deficient in CD73. CD73$^{-/-}$ mice developed markedly more severe colitis and exhibit unresolved inflammation and produce high levels of the proinflammatory cytokines IL-1β and TNF-α compared to WT mice. Colonic epithelial cells from CD73$^{-/-}$ mice show increased TLR-9 and NF-κB activity and have decreased expression of several tight-junction-associated proteins when compared to WT mice, along with an associated increase in gastrointestinal permeability. From these findings, we hypothesize that CD73-generated adenosine is a key modulator in gut inflammation and IBD and mediates its effects through regulating inflammation and maintaining epithelial barrier integrity.

2. Materials and Methods

2.1. Mice. C57BL/6 WT and *rag*$^{-/-}$ mice were purchased from Jackson Laboratories. CD73$^{-/-}$ mice were obtained from Dr. Linda Thompson at the Oklahoma Medical Research Foundation [23]. Mice were bred and housed under specific pathogen-free conditions in the mouse facility at Cornell University. All procedures performed on mice were approved by the Cornell University IACUC. All mice used in these experiments were between 8 and 10 weeks old.

2.2. DSS-Induced Experimental Colitis. 3% Dextran Sodium Sulfate (DSS, MW = 36,000–50,000) was administered in drinking water to experimental animals [24]. Age-matched males and females WT and CD73$^{-/-}$ were used in these experiments. Weight loss was normally observed 4-5 days after the initiation of DSS treatment, and experiments were halted after animals lost ~20% of initial body weight.

2.3. Passively Induced IBD. CD4$^+$ cells were isolated from processed spleens and lymph nodes of naïve WT and CD73$^{-/-}$ mice by negative selection using magnetic beads (Invitrogen). CD4$^+$ cells were further subdivided into CD4$^+$CD45RB^{high}CD25$^-$ and CD4$^+$CD45RB^{low}CD25$^+$ populations using a BD-Biosciences FACS Aria system. Two million CD4$^+$CD45RB^{high} cells from either WT or CD73$^{-/-}$ mice were injected into *rag*$^{-/-}$ mice. Some mice additionally received 2×10^6 CD4$^+$CD45RB^{low}CD25$^+$ cells isolated from either WT or CD73$^{-/-}$ mice. Mice were weighed daily for 45 days to track disease progression.

2.4. Isolation of Primary Colonic Epithelial Cells. Whole colons were isolated, the lumen exposed by a longitudinal cut, washed with Ca^{++}Mg^{++} free HBSS + 2% FBS, cut into 1-2 mm^2 sections, and washed 2X with Ca^{++}Mg^{++} free HBSS + 2% FBS. The tissue was then incubated for 30 min at 37° C in Ca^{++}Mg^{++} free HBSS + 10% FBS + 1 mM EDTA + 1 mM DTT + penicillin/streptomycin. The digest was filtered through a 70 μm mesh and spun at 200 ×g for 10 min. Primary colonic epithelial cells include resident intraepithelial lymphocytes (iELs), as no Percoll gradient was used to separate this population.

2.5. Flow Cytometry. Cells were isolated either from the spleen, mesenteric lymph nodes, colonic epithelium, or colonic patches of mice and processed into a single-cell suspension. These cells were subsequently stained with rat anti-mouse CD4, CD25 (BD Biosciences), CD73, and Foxp3 or hamster anti-mouse CD11c (eBioscience) at a 1 : 250 dilution in PBS. After incubating for 30 min at 4°C with the antibody cocktail, the cells were washed and resuspended in PBS. FoxP3 staining was performed using the eBioscience FoxP3-APC kit. Sample data were acquired using a FACScalibur with CellQuest software. Data files were analyzed using FlowJo software.

2.6. Histology. Hematoxylin and eosin (H&E) staining was performed by the Cornell University College of Veterinary Medicine Core Histology lab on formalin fixed tissue samples. Immunohistochemistry and immunofluorescence were performed on OCT embedding tissue snap-frozen in liquid nitrogen-chilled methylbutane and sectioned into 5 μm slices on a cryostat. Primary antibodies used were CD4, CD45, CD11c (hamster anti-mouse 1 : 30), and CD321 (JAM-A, rat anti mouse, 1 : 100). Secondary antibodies used were biotinylated goat anti-rat IgG and biotinylated goat anti-hamster IgG (1 : 100). For immunohistochemistry, slides were treated with streptavidin-HRP followed by AEC and counterstained with hematoxylin. Immunofluorescence staining for CD321 was performed by treating with a 1 : 200 dilution of streptavidin-AF488 for 90 minutes at 37°C and then fixed in 1% formalin for 10 minutes.

2.7. Clinical Score and Histological Analysis. Changes in body weight were assessed as a percent change in body weight compared to baseline. A scoring system (from 0–4) was used to determine the presence of occult or overt blood

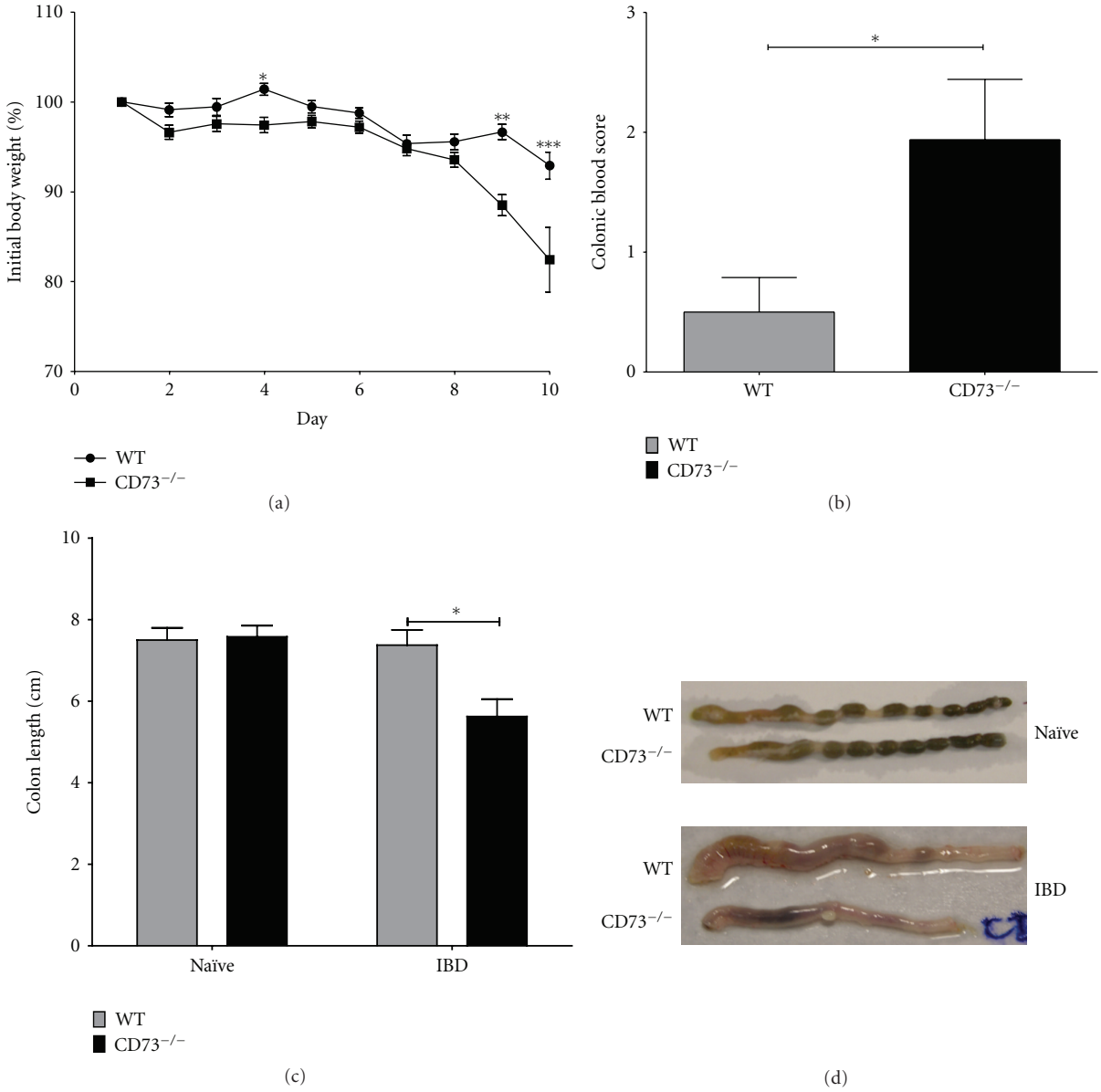

Figure 1: CD73$^{-/-}$ mice develop more severe clinical signs of IBD than WT mice. (a) WT (circles) and CD73$^{-/-}$ (squares) mice were given 3% DSS in drinking water and weighed daily for 10 days. Results are represented as % of initial weight (±SEM, summary of 5 experiments, $n = 26$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$). (b) Mean (±SEM, $n = 4$, $^{*}P < 0.05$, representative of 5 experiments) colonic blood score is displayed (0 = no blood in colon; 1 = blood in 25% of colon; 2 = blood in 50% of colon; 3 = blood in 75% of colon; 4 = blood in 100% of colon). (c) Mean (±SEM, $n = 4$, $^{*}P < 0.05$, representative of 5 experiments) colon length (cm) is displayed, along with (d) a representative photograph.

in the stool. Colonic inflammatory score was determined by the extent of inflammatory cell infiltrates (0–3) and tissue damage (0–3). Scoring was performed in a blinded manner. Cell infiltration and tissue damage were added and the combined "colitis severity score" ranged from 0 to 6 (description in legends to Figures 2, 3 and 4).

In Figure 2, (a) histology of colons from WT (top panels) and CD73$^{-/-}$ (bottom panels) mice treated with 3% DSS in drinking water. Left two panels show hematoxylin and eosin (H&E) stains displaying colonic epithelial crypt histopathology and appearance of granulomas (in CD73$^{-/-}$) mice. Right three panels show CD4^{+} cell (brown) infiltration in the colon. (b) Mean (±SEM, $^{****}P < 0.001$, $n = 8$) colonic inflammatory score is displayed. The scoring scale is comprised of two components: (i) inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2, and transmural extension of the infiltrates = 3); (ii) tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2 and extensive mucosal damage and extension through deeper structures

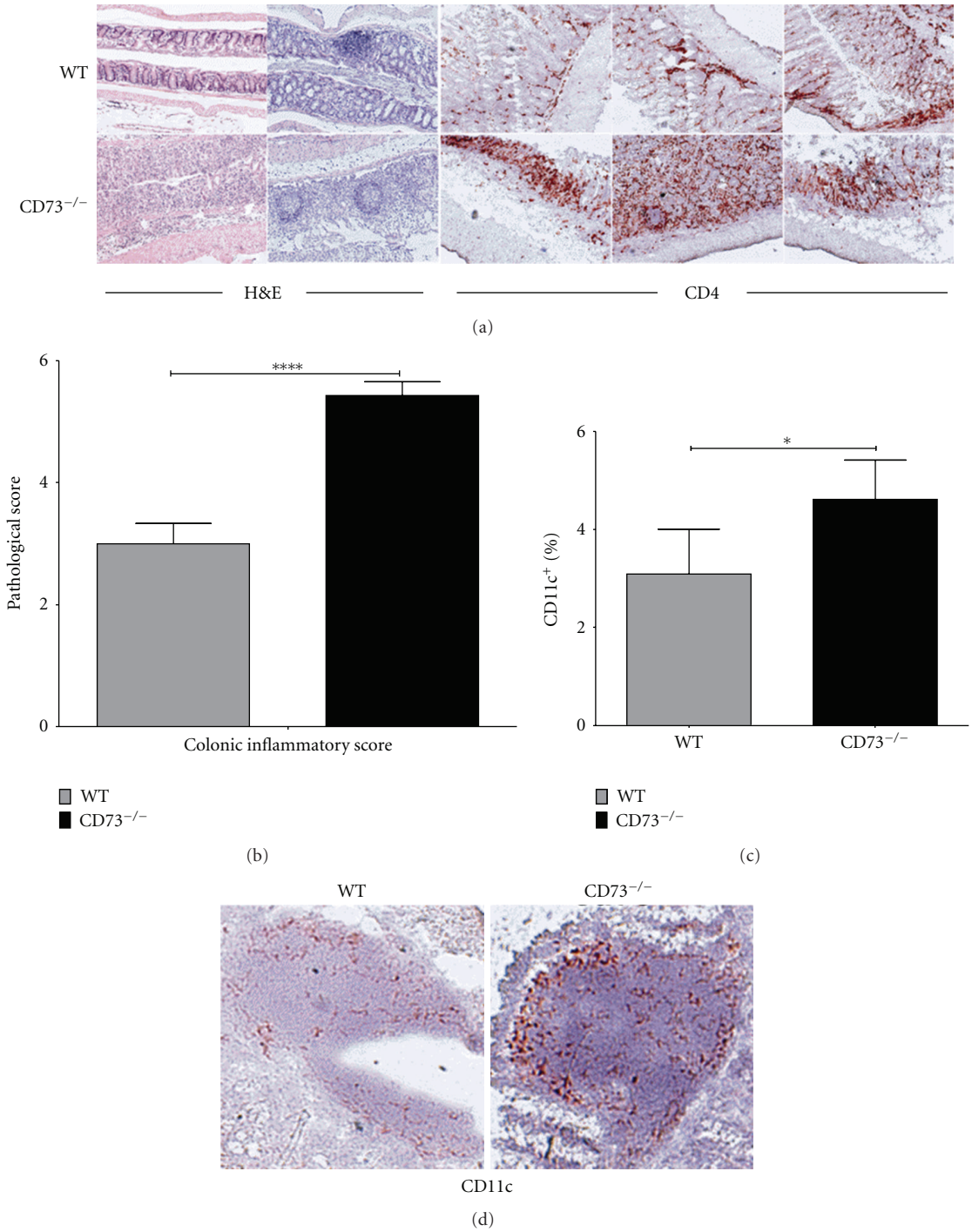

Figure 2: CD73$^{-/-}$ mice display a higher degree of infiltration in the colon compared to WT mice.

of the bowel wall = 3). The two scores (cell infiltration and cell damage) are added, and the combined histological colitis severity score ranges from 0 to 6. (c) Colonic patches from WT and CD73$^{-/-}$ mice treated with 3% DSS in drinking water were isolated and stained for CD11c and analyzed by FACS. Results are displayed as % of CD11c^{+} cells (data representative of 3 experiments). (d) Immunohistochemistry of colons from WT (left) and CD73$^{-/-}$ (right) mice treated with 3% DSS in drinking water. Sections show CD11c infiltration in the colons of mice. (e) IL-1β and TNF-α produced by lymphocytes from colonic patches of CD73$^{-/-}$ and WT mice after 24-hour incubation in medium without stimulation. Samples were analyzed by Bio-Plex instrument (from three different experiments, $n = 15$ mice, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, ND = not detected).

In Figure 3, (a) CD4^{+}CD45RB^{high} T cells from WT (circles) and CD73$^{-/-}$ (squares) were transferred into *rag*$^{-/-}$ mice, which were monitored for 45 days. Results

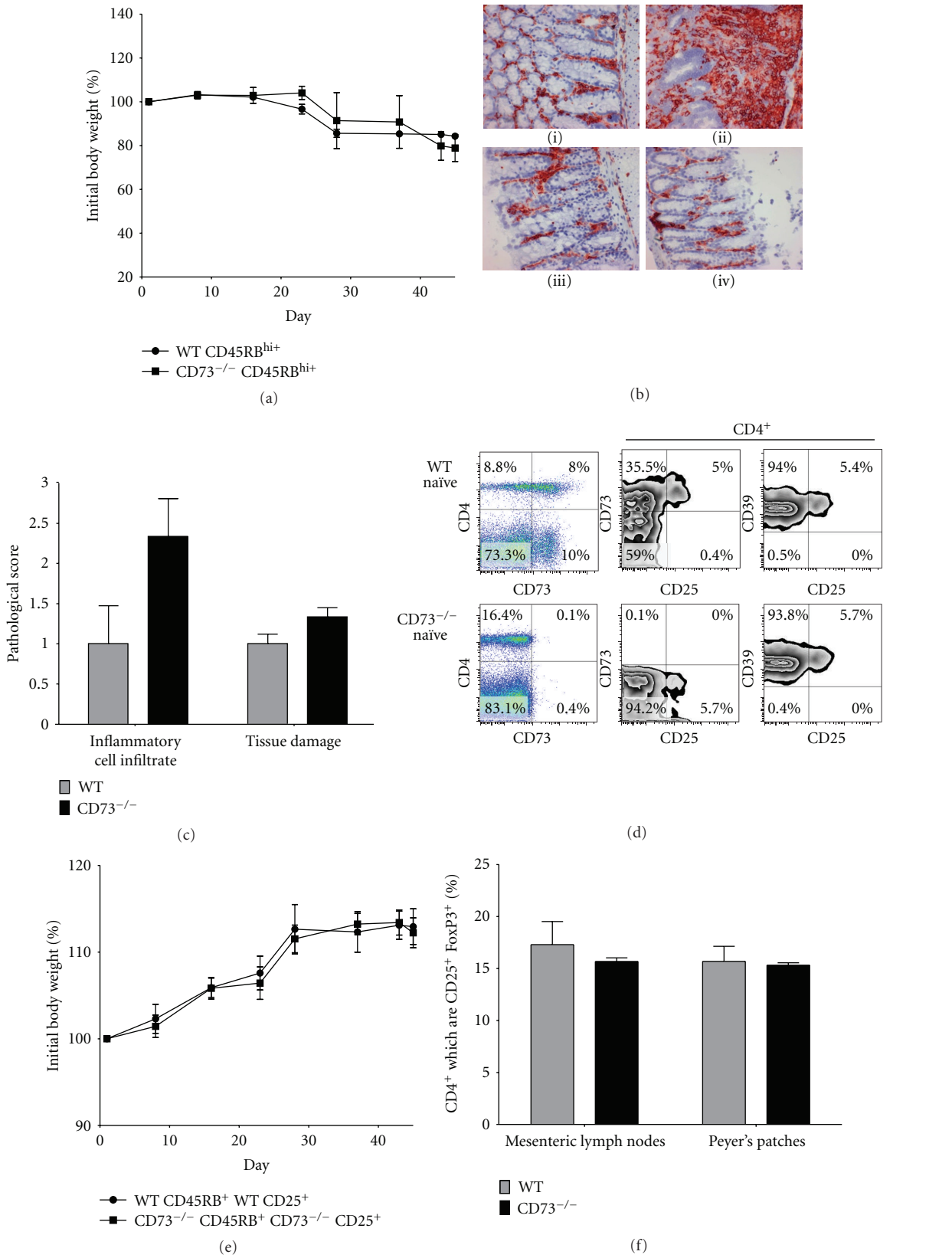

FIGURE 3: CD4+ T cells from CD73−/− mice transfer a similar degree of colitis compared to WT mice and show no defect in T_{reg} suppression of IBD.

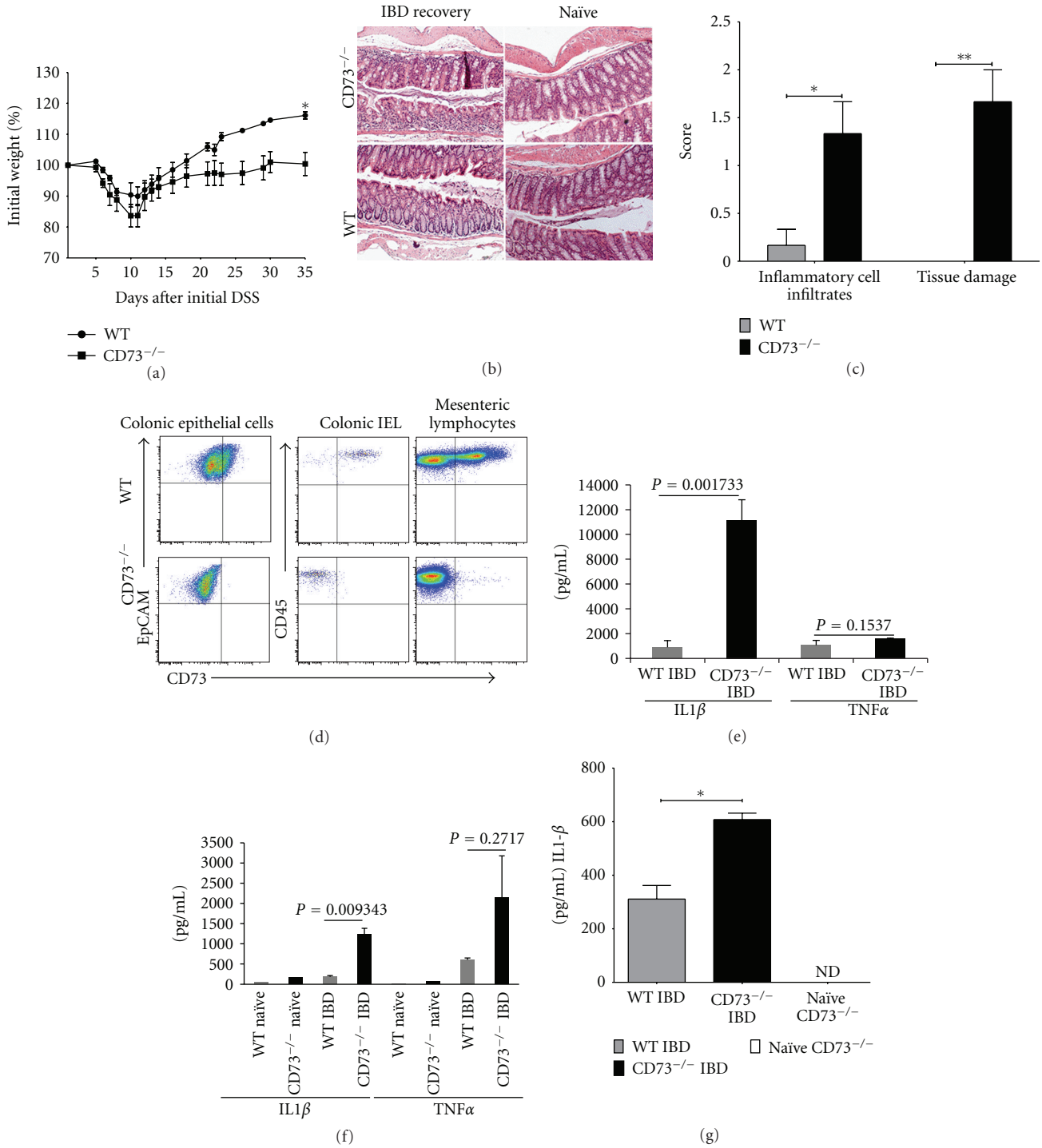

Figure 4: CD73$^{-/-}$ mice exhibit ongoing inflammation and slower recovery from IBD compared to WT mice. (a) WT (circles) and CD73$^{-/-}$ (squares) mice were given 3% DSS in drinking water and weighed daily for 35 days. DSS was removed at day 8 and replaced with drinking water. Results are represented as % of initial weight (±SEM, $^{*}P < 0.05$, one of two representative experiments). (b) H&E staining of colons from CD73$^{-/-}$ (top) and WT (bottom) mice taken on day 35 (left) compared to naïve (right) mice. (c) Mean (±SEM, $^{*}P < 0.05$, $^{**}P < 0.01$) colonic inflammatory score is displayed ($n = 5$ mice). The scoring scale is comprised of two components: (i) inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2, and transmural extension of the infiltrates = 3); (ii) tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2, and extensive mucosal damage and extension through deeper structures of the bowel wall = 3). (d) FACS analysis of CD73 expression on colonic epithelial cells from naïve WT (top left panel, 28%) and CD73$^{-/-}$ (bottom left panel, 0%) mice. Colonic epithelial cells identified with CD326/EpCam. Colonic IEL's identified with CD45 (middle panels, WT 87% CD73^{+}). Mesenteric lymph nodes shown as a positive control (right panels, WT 39% CD73^{+}). (e) IL-1β and TNFα production in colon preparation isolated from WT and CD73$^{-/-}$ mice on day three of DSS treatment and (f) day eight of DSS treatment. (g) Colons isolated from WT and CD73$^{-/-}$ mice on day 22 after removal of DSS were cultured (in medium alone) for 24 hours and supernatant analyzed by Bio-Plex for IL-1β. Results (mean ±SEM, $^{*}P < 0.05$, representative of one experiment, $n = 3$ mice per group) are presented as pg/mL IL-1β produced.

Table 1

Target gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
JAM-A	TCTCTTCACGTCTATGATCCTGG	TTTGATGGACTCGTTCTCGGG
Claudin 2	GTACCCTTTTAGGACTTCCTGC	CCCACCACAGAGATAATACAAGC
A Catenin	TCTCTACTGCCACCAGCTCAAC	AAGCCATCCCCTGTGACTTCT
GAPDH	CCCCAATGTGTCCGTCGTG	GCCTGCTTCACCACCTTCT
TLR1	GTCTCCCCACTTCATCCAGA	GCTTGTTCTTCTCTGTGGGC
TLR3	GGTCCCCAGCCTTCAAAGAC	ACGAAGAGGGCGGAAAGGT
TLR4	CAGGTGGAATTGTATCGCCT	CGAGGCTTTTCCATCCAATA
TLR5	CCACCGAAGACTGCGATGA	GTGACCGTGCACAGGATGAA
TLR9	ATG GTT CTC CGT CGA AGG ACT	GAG GCT TCA GCT CAC AGG G
PGK1	CTC CGC TTT CAT GTA GAG GAA G	GAC ATC TCC TAG TTT GGA CAG TG

(5 mice/group, mean ± SEM, representative of 3 experiments) are displayed as % of initial weight. (b) CD45 staining of frozen colons from $rag^{-/-}$ mice 45 days after T cell transfer: (i) WT $CD45RB^{+}$ donor, (ii) $CD73^{-/-}$ $CD45RB^{+}$ donor, (iii) WT $CD45RB^{+}$ and WT $CD25^{+}$ donors, and (iv) $CD73^{-/-}$ $CD45RB^{+}$ and $CD73^{-/-}$ $CD25^{+}$ donors. (c) Colonic inflammatory score is displayed with separate scores for inflammatory cell infiltrate (focus of inflammatory cells in the lamina propria = 1, confluence of inflammatory cells = 2 and transmural extension of the infiltrates = 3), and tissue damage (discrete lymphoepithelial lesions = 1, mucosal erosion = 2, and extensive mucosal damage and extension through deeper structures of the bowel wall = 3) ($n = 6$ mice, inflammatory cell infiltrate, ±SEM, $P = 0.05$, tissue damage, $P = 0.4$). (D) CD25, CD73, and CD39 expression on $CD4^{+}$ T cells from mesenteric lymph nodes and colonic patches of naïve WT and $CD73^{-/-}$ mice. Left-hand panels show distribution of CD73 on $CD4^{+}$ cells. Middle panels show distribution of iELs, and right panels are gated on $CD4^{+}$ cells and show distribution of CD25/CD73 and CD25/CD39 expression. (e) $CD4^{+}CD45RB^{high}$ and $CD4^{+}CD25^{+}$ ($CD45RB^{lo}$) T cells from WT (circles) or $CD73^{-/-}$ (squares) were transferred into $rag^{-/-}$ mice. Results (mean ± SEM, representative of 3 experiments) are displayed as % of initial weight. (f) FoxP3 expression in colonic patches and mesenteric lymph nodes from $CD73^{-/-}$ versus WT mice (representative of two experiments).

2.8. Cytokine Analysis. For Figure 2, colonic patches were cultured overnight at 37°C in Bruff's media. For Figure 4, whole colons were isolated from DSS-treated and naïve $CD73^{-/-}$ and WT animals and cultured overnight at 37°C in Bruff's media. The culture supernatant was isolated and frozen at −80°C. Analysis of cytokine levels was performed using a Bio-Plex system.

2.9. Real-Time PCR. All data was generated using either the Thermo Scientific SYBR Green kit and the ABI 7500 Real-Time PCR machine or the Kapa SYBR FAST qPCR kit and the Bio-Rad C1000 Real-Time PCR machine. Expression of all genes was standardized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and relativised to WT expression of the gene in question or was compared to PGK1 (housekeeping gene) expression. Primers, whose sequences are shown in Table 1, were obtained from IDT.

2.10. Gastrointestinal Permeability. Briefly, mice were fasted for 3 hrs and were rectally administered 200 μL of 10 mg/mL (0.1 mg/g body weight) 10 kDa FITC-dextran (Invitrogen) in PBS [25]. After 1 hour, mice were bled via their submandibular vein, the whole blood spun at 2,000 ×g for 5 min, and the serum isolated. This isolation yielded approximately 150 μL of serum per animal. Serum fluorescence was measured on a BioTek (Winooski, VT) Synergy 4 analyzer. FITC-Dextran concentration was calculated from standard curves generated by serially diluting FITC-Dextran.

2.11. Western Blotting. Western blot analysis as described in [26] was performed on colonic epithelial cells of $CD73^{-/-}$ and WT mice. Briefly, cells (2×10^6) were lysed in reducing SDS sample buffer followed by centrifugation at 18,000 g for 5 min. Samples were boiled for 10 minutes after which equal amounts of total cell lysates were separated by 10% SDS-PAGE and electrophoresed onto PVDF (Millipore, Bedford, MA). Samples were incubated with Ab specific for dual-phosphorylated (active) NF-κB P65 subunit used at the concentrations recommended by the manufacturer (Cell Signaling Technology, Beverly, MA) in 5% BSA in Tris buffered saline, 0.1% Tween20 overnight at 4°C.

3. Results

3.1. $CD73^{-/-}$ Mice Develop More Severe IBD Than WT Mice. Since CD73 catalyzes the formation of extracellular immunosuppressive adenosine from damaged cells during inflammation and is highly expressed on colon tissue [16–19], we investigated the impact of a lack of CD73 in the intestinal mucosa. To do this, we induced IBD in $CD73^{-/-}$ and WT mice by oral treatment with 3% DSS in drinking water. DSS-induced colitis resulted in significant weight loss, epithelial damage, and robust colonic inflammation (Figure 1). Daily monitoring of IBD revealed that $CD73^{-/-}$ mice developed markedly more severe clinical signs of IBD, including diarrhea and dehydration (data not shown), when compared to WT mice (Figure 1). Frequently, disease had to be aborted before the duration of the 10

day protocol because greater than 50% of CD73$^{-/-}$ mice exhibited substantial weight loss and severe rectal bleeding. This was accompanied by more pronounced weight loss (Figure 1(a)), higher colonic blood scores (Figure 1(b)), and shorter colon length (Figure 1(c)).

Histological analyses of the colons of DSS-treated CD73$^{-/-}$ mice revealed extensive destruction of colonic epithelial architecture, including epithelial crypt destruction, massive immune cell infiltration into the lamina propria, and the appearance of granulomas (in 30% of mice), and an overall increase in colon inflammatory score compared to WT mice (Figures 2(a) and 2(b)). These immune cell infiltrates consist of CD45^{+} cells (a general marker for leukocytes, data not shown) with a substantial number being CD4^{+} T cells (Figure 2(a)). FACS analysis of colonic patches (lymphoid aggregates from colon) also revealed a higher percentage of CD11c^{+} dendritic cells in CD73$^{-/-}$ mice compared to WT mice (Figures 2(c) and 2(d)).

We next determined the cytokine profile of infiltrating leukocytes isolated from the colon of DSS-treated CD73$^{-/-}$ and WT mice. Cells from colonic patches of CD73$^{-/-}$ mice produced higher levels of IL-1β compared to those from WT mice (data not shown). There was no significant difference in TNF-α, IFN-γ, IL-17, IL-4, or IL-10 levels between WT and CD73$^{-/-}$ mice (data not shown). The increase in IL-1β suggests that infiltrating inflammatory cells into the colon are capable of contributing to the severe IBD in CD73$^{-/-}$ mice.

3.2. CD45RB^{high}CD4^{+} Cells from CD73$^{-/-}$ Mice Transfer Similar Degree of Colitis as WT Mice. Due to the immunoregulatory effects of localized CD73-driven adenosine production, we next determined if the severe IBD phenotype observed in DSS-treated CD73$^{-/-}$ mice was due to a more proinflammatory nature of the CD4^{+} T-cell population. To address this question, we induced IBD by transferring CD4^{+}CD45RB^{high} T cells into *rag*$^{-/-}$ recipients [27]. IBD induced by this method involves differential activation of T_H-1 cells and can be inhibited by cotransfer of CD4^{+}CD45RB^{low} (consisting of CD25^{+} T regulatory cells) cells into *rag*$^{-/-}$ recipients [28]. CD4^{+}CD45RB^{high} cells were isolated from spleens of naïve WT or CD73$^{-/-}$ mice and adoptively transferred into *rag*$^{-/-}$ recipients. Recipients of CD73$^{-/-}$ CD4^{+}CD45RB^{high} T cells exhibited a similar weight loss pattern to those recipients of WT CD4^{+}CD45RB^{high} T cells (Figure 3(a)). Histological analysis of colons revealed *rag*$^{-/-}$ mice that received CD4^{+}CD45RB^{high} cells from CD73$^{-/-}$ mice had significantly more immune cell infiltration in the lamina propria compared to recipients of CD4^{+}CD45RB^{high} cells from WT mice (Figures 3(b) and 3(c)). However, the resultant colonic epithelial damage compared between recipients of WT and CD73$^{-/-}$ CD4^{+}CD45RB^{high} donor cells is not statistically significant (Figures 3(c) and see S1 in supplementary material available online at doi:101155/2012/260983). This suggests that the inflammatory immune cells infiltrating the large intestine is not the sole cause of the very severe IBD observed in CD73$^{-/-}$ mice.

Recent studies have indicated that CD73 and CD39 are important for immune suppression by T regulatory cells [20]. We therefore asked whether CD4^{+} T regulatory (T_{reg}) cells from CD73$^{-/-}$ mice were defective in their ability to suppress IBD. In naïve WT mice, fifty percent of CD4^{+} T cells express CD73 (Figure 3(d)) and all CD4^{+}CD25^{+} cells express both CD73 and CD39 (Figure 3(d)). CD4^{+}CD45RB^{high} and CD4^{+}CD25^{+} (CD45RB^{lo}) cells from spleens of naïve WT mice were cotransferred into *rag*$^{-/-}$ recipients. Similarly, naïve CD4^{+}CD45RB^{high} and CD4^{+}CD25^{+} (CD45RB^{lo}) cells from CD73$^{-/-}$ mice were cotransferred into *rag*$^{-/-}$ recipients. *Rag*$^{-/-}$ mice that received CD73$^{-/-}$ CD4^{+}CD45RB^{high} and CD4^{+}CD25^{+} cells developed no clinical signs of IBD and showed a similar weight-gain pattern to *rag*$^{-/-}$ mice receiving WT CD4^{+}CD45RB^{high} and CD4^{+}CD45RB^{lo}CD25^{+} cells (that also showed no signs of IBD) (Figure 3(e)). Further, similar to WT mice, CD73$^{-/-}$ mice harbor similar frequency of CD4^{+} FoxP3^{+} T cells in colonic patches (lymphoid follicles) and mesenteric lymph nodes compared to those of WT mice (Figure 3(f)) (also in peripheral lymphoid organs [29]). These results indicate that neither the numbers nor the function of CD4^{+}Treg cells from CD73$^{-/-}$ mice is significantly altered and thus is not the only contributing factor in the severe IBD in CD73$^{-/-}$ mice. From these studies we conclude that a lack of CD73 expression on CD4^{+}T_{reg} cells did not majorly hamper their ability to suppress IBD.

3.3. CD73$^{-/-}$ Mice Exhibit Ongoing Inflammation and Slower Recovery Than WT Mice. Under normal conditions, WT mice can recover from colitis once DSS treatment is removed [24]. To determine whether CD73$^{-/-}$ mice can recover from DSS-induced colitis we replaced DSS with drinking water on day eight in both WT and CD73$^{-/-}$ mice and monitored their body weight as an indication of disease alteration. CD73$^{-/-}$ mice recovered at a slow rate following the removal of DSS from their drinking water, regaining their initial body weight 22 days after DSS removal (Figure 4(a)). This was in sharp contrast to WT mice that regained their initial body weight seven days after the removal of DSS and gained an additional 20% of their initial body weight by 22 days after T DSS removal (Figure 4(a)). Examination of colon pathology of WT mice after day 22 showed full recovery: colonic architecture showed little to no cell infiltration, no tissue damage, and normal looking epithelial crypts and goblet cells (Figures 4(b) and 4(c)). While CD73$^{-/-}$ mice (day 22) exhibited significantly reduced colonic pathology compared to that observed during DSS treatment, they still show modest cell infiltration, some epithelial hyperplasia, and noticeable crypt destruction (Figures 4(b) and 4(c)), indicating prolonged or ongoing inflammation illustrated by the colonic inflammatory score (Figure 4(c)).

3.4. Increased IL-1β and TNF-α Production in Colonic Epithelium of CD73$^{-/-}$ Compared to WT Mice. While inflammatory immune cells lacking CD73 play an important role in IBD pathogenesis in CD73$^{-/-}$ mice (Figure 3(b)), it is apparent that they are not the only contributing factor that result in the very severe IBD. We next focused our attention on the colonic epithelium that is comprised of a single layer of intestinal epithelial cells that forms a physical as well as

a chemical barrier between the host and the microbial community [30]. FACS analysis of colonic epithelial cells from naïve WT mice, double stained with an epithelial marker plus CD73, showed that about 40% of epithelial cells expressed CD73 (including intraepithelial lymphocytes (iELs) which make up about 10% of $CD73^+$ cells) (Figure 4(d)).

We next focused on the colon in $CD73^{-/-}$ mice. We reasoned that the lack of CD73 expression on the colonic epithelium must contribute significantly to the severe IBD, since the immune cells lacking CD73 cannot recapitulate the severe colitis in $CD73^{-/-}$ mice. The intestinal epithelium closely interacts with underlying mucosal immune cells to control the immune response [2, 31]. The intestinal epithelium and its products determine whether the underlying immune status is suppressive or inflammatory. Damage or activation of the epithelial barrier results in production of inflammatory mediators by immune cells and epithelial cells [8]. We hypothesized that the lack of CD73 expression on colonic epithelial cells might contribute to the heightened proinflammatory conditions in IBD in $CD73^{-/-}$ mice. We isolated colons of WT and $CD73^{-/-}$ mice over the course of DSS treatment, including the period after DSS removal, cultured them in media overnight, and analyzed supernatants for cytokines by ELISA. We focused specifically on IL-1β and TNF-α, as these cytokines are implicated in colitis both in mice and humans [32]. Further, extracellular adenosine is a potent regulator of proinflammatory cytokines including IL-1β and TNF-α [14]. Colons from $CD73^{-/-}$ mice produced dramatically higher levels of IL-1β (more than 10-fold) and with slight increase in TNF-α on day 3 of DSS treatment compared to WT (Figure 4(e)). By day 8 of DSS treatment, $CD73^{-/-}$ mice consistently produced significantly higher levels of these cytokines compared to WT mice (Figure 4(f)). Furthermore, supernatants from colons isolated after DSS removal (day 22) show significantly higher levels of IL-1β (similar to day 8 of DSS, but not TNF-α) observed in $CD73^{-/-}$ mice (Figure 4(g)). IL-1β and TNF-α are potent proinflammatory factors that stimulate proliferation, apoptosis, phagocytosis, and transmigration of cells across epithelial and endothelial barriers as well as activate innate receptors on innate immune and epithelial cells [33]. The increased production of IL-1β and TNF-α is tightly correlated with IBD severity and tissue destruction in animals and human patients [1] and is consistent with the severe colitis and apparent lack of resolution of the inflammatory process in $CD73^{-/-}$ mice.

3.5. Increased Activation of NF-κB in $CD73^{-/-}$ Mice Compared to WT Mice. IL-1β induces the phosphorylation of IκB resulting in its degradation and the activation of NF-κB [34]. The constitutive activation of NF-κB is associated with aberrant expression of proinflammatory genes induced by cytokines such as IL-1β and is linked to the pathogenesis of IBD in both human and mice [35]. Adenosine is a potent regulator of IL-1β, and IL-1β can increase the expression of the A_{2A} adenosine receptor [36]. Further, adenosine is a negative regulator of NF-κB activation in human intestinal epithelial cells [36]. Thus, we hypothesize that constitutive production of IL-1β in $CD73^{-/-}$ mouse colonic epithelia (including intraepithelial lymphocytes) even after DSS removal may cause constitutive activation of NF-κB. To investigate this, we isolated colonic epithelial cells from WT and $CD73^{-/-}$ mice over the course of IBD and tested them for presence of NF-κB activity. Figure S2 shows that, at various disease intervals, NF-κB p65 protein is augmented in colonic epithelial cells in $CD73^{-/-}$ compared to WT mice. These findings suggest that the lack of extracellular adenosine may result in prolonged NF-κB activity after the inflammatory trigger (DSS) is removed. This inflammatory program, through constitutive production of IL-1β, can promote a proinflammatory feedback loop and a vicious inflammatory cycle.

3.6. Increased Intestinal Inflammation Is Associated with Increased Intestinal Permeability and Decreased Expression of Tight-Junction Associated Proteins in the Colons of $CD73^{-/-}$ Mice. In IBD patients and animal models of IBD, increased inflammation strongly correlates with an increase in intestinal permeability and vice versa [37, 38]. To determine if intestinal barrier permeability is altered, we examined the barrier integrity in $CD73^{-/-}$ and WT mice by treating mice rectally with FITC-Dextran and measuring its concentration in sera one hour later. As shown in Figure 5(a), $CD73^{-/-}$ mice exhibit significant increase in gastrointestinal permeability compared to WT. The protective barrier function of the intestinal epithelium is regulated by intercellular tight junctions that seal the space between epithelial cells [31]. Tight junction molecules are comprised of occludins, claudins, and JAM proteins [31]. Further, JAM-A/JAM-1 has been shown to be critical in the maintenance of epithelial barrier permeability, as $JAM\text{-}A^{-/-}$ mice have increased leukocyte infiltration and increased mucosal barrier permeability to macromolecules in the intestinal mucosa [39]. We analyzed mRNA transcript levels of several junction molecules that are associated with tight-junction stability to determine if the loss of these factors could be responsible for the destruction of the colonic architecture observed in DSS-treated $CD73^{-/-}$ mice. We observed a significant decrease in the mRNA levels of JAM-A, α-Catenin, and Claudin 2 in the colons of $CD73^{-/-}$ mice compared to WT mice (Figure 5(b)). Furthermore, immunofluorescence staining with anti-JAM-A antibody shows JAM-A protein expression is decreased at the borders of epithelial cells in naïve $CD73^{-/-}$ mice, while expression is localized to the plasma membrane in naïve WT mice (Figure 5(c)). These findings further demonstrate that extracellular adenosine is important in regulation and maintenance of intestinal barrier integrity.

3.7. TLR9 Is Upregulated in Colons of $CD73^{-/-}$ Mice during IBD. To further evaluate the status of the colonic epithelia and their ensuing inflammatory state, we next focused on Toll-like receptors (TLRs) [40]. We hypothesize that the ongoing inflammation in $CD73^{-/-}$ mice is due to a breakdown of the intestinal epithelial barrier that leads to leakage of luminal antigens into the lamina propria and activation of innate receptors. Intestinal epithelial cells express a spectrum of Toll-like receptors (TLRs), recognize and respond to microbial products (MAMP/PAMPS), and

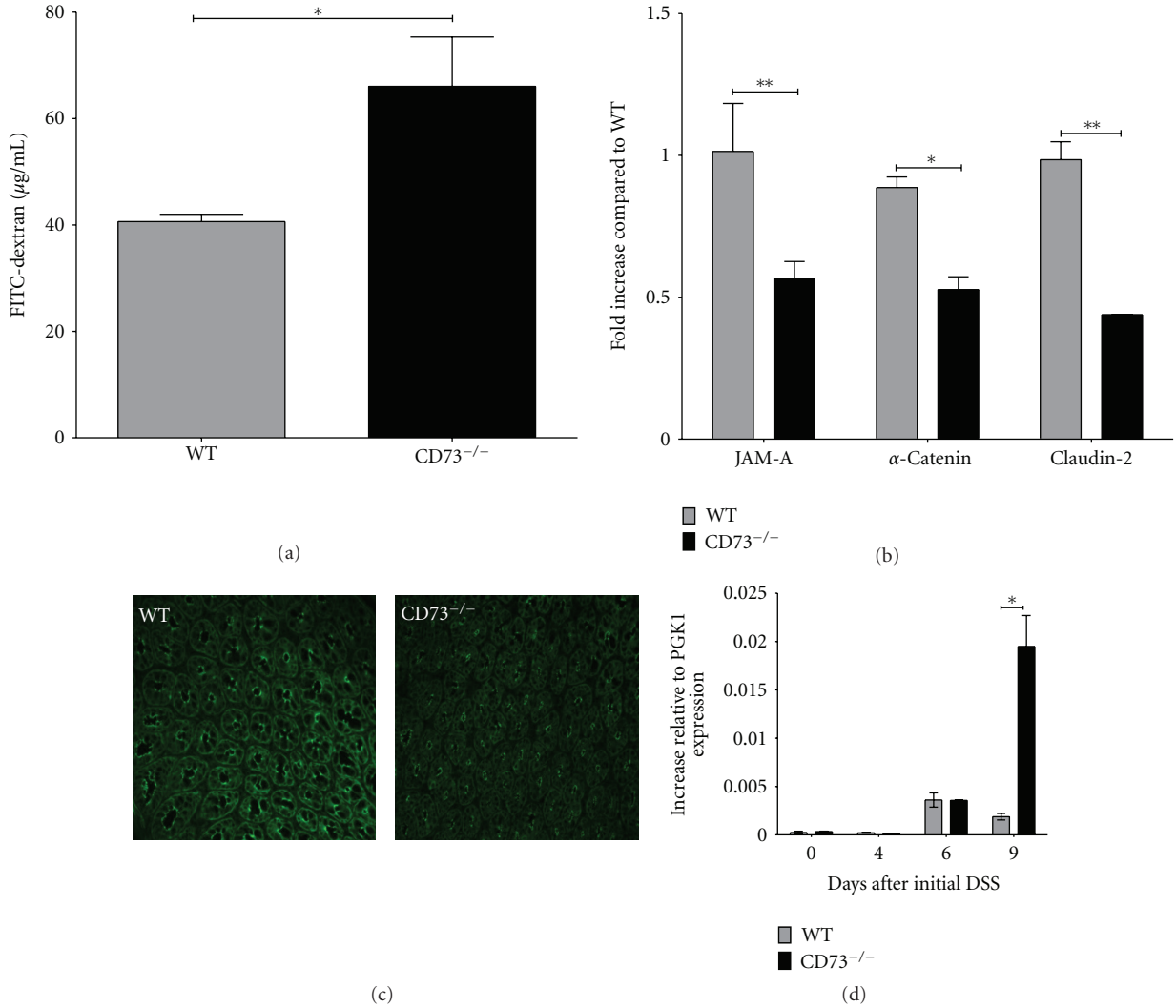

Figure 5: Upregulation of TLR9 in CD73$^{-/-}$ decreased expression of tight-junction associated proteins observed in the colons of CD73$^{-/-}$ mice compared to WT mice. (a) Colonic permeability was determined in WT and CD73$^{-/-}$ mice. Serum concentrations of FITC-Dextran (MW 10,000) in WT (gray bar) and CD73$^{-/-}$ (black bar) mice were obtained following rectal administration of 10 KDa FITC-Dextran (0.5 mg/Kg) and measuring FITC-Dextran in the blood 1 hour later. Results are expressed as mean ±SEM, $n = 5$, $^{*}P < 0.05$). Results are expressed as (mean ±SEM, $n > 3$, $^{*}P < 0.05$) relative fluorescence compared to WT. (b) Colons of WT (gray bars) and CD73$^{-/-}$ (black bars) were analyzed for mRNA expression of the adhesion molecules JAM-A, αCatenin, and Claudin-2. Results are expressed as (mean ±SEM, $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$) fold increase in CD73$^{-/-}$ compared to WT. (c) Fluorescent microscopy of colons from naïve WT (left panel) and CD73$^{-/-}$ (right panel) mice stained for JAM-A (green). (d) Colons were isolated from CD73$^{-/-}$ or WT mice over the course of IBD and analyzed for TLR9 mRNA expression by real-time PCR. Results are presented as TLR9 expression level relative to the housekeeping gene PGK1. (Representative of 3 experiments.)

can trigger inflammatory responses by mucosal immune cells [41]. To determine if the lack of CD73 expression on the colon results in increased expression of TLRs, we analyzed colons of IBD WT and CD73$^{-/-}$ mice by quantitative PCR analysis for mRNA expression. WT mice expressed background mRNA levels of a few TLRs, while other TLRs are not detected (Figure S3). In stark contrast, CD73$^{-/-}$ mice express more than 10-fold higher levels of TLR9 and just above background levels of TLR 3 and 5 (Figure S3). To determine the onset of TLR9 expression over the course of DSS treatment, we analyzed colons at different time points during IBD. We observed consistent increase in TLR9 expression in the latter stages of IBD (Figure 5(d)). These findings strongly support the idea of a more activated and proinflammatory state of the colonic epithelium in CD73$^{-/-}$ mice compared to WT mice.

4. Discussion

The goal of this study was to determine the role of extracellular adenosine in the regulation of intestinal inflammation. We demonstrate that mice lacking CD73 (and thus unable to adequately synthesize extracellular adenosine) exhibit severe

colitis induced by DSS treatment. This finding is consistent with previous studies that showed CD73$^{-/-}$ mice presented a more severe IBD induced by trinitrobenzene sulfonate (TNBS) administration than their WT counterparts [11]. In this study, CD73$^{-/-}$ mice were found deficient in INF-α, which may be important for regulating colonic inflammation. Here, we demonstrate that CD73$^{-/-}$ mice are unable to resolve inflammation even after the removal of DSS. Compared to WT mice, colonic epithelia from CD73$^{-/-}$ mice produced consistently high levels of IL-1β and TNF-α that correlate with increased NF-κB and TLR9 expression and resultant gastrointestinal permeability. In this study, we demonstrate that heightened inflammation, induced by aggressive inflammatory immune cells, activated inflammatory colonic epithelial cells and increased intestinal barrier permeability result in severe unresolved DSS-induced colitis in mice lacking CD73. Similar outcomes from previous studies demonstrate that CD73$^{-/-}$ mice presented more severe IBD, while these two studies use different approaches.

CD73$^{-/-}$ mice exhibit massive immune cell infiltration into the colon. The infiltrating cells are comprised primarily of CD4^{+} T cells and dendritic cells. These CD4^{+} T cells in CD73$^{-/-}$ mice produced high levels of the proinflammatory cytokines IL-1β and TNF-α, which are known to play a major role in the pathogenesis of IBD in humans and mice. However, adoptive transfer of naïve CD4^{+}CD45RB^{high} (inducer) T cells from CD73$^{-/-}$ mice into naive *rag*$^{-/-}$*cd73*$^{+/+}$ recipients shows that while these mice developed severe colitis, the extent of the tissue damage was not dramatically different when compared to recipients that received CD4^{+}CD45RB^{high} T cells from WT mice. These findings suggest that the infiltrating immune cells are not the only cause of the severe IBD in CD73$^{-/-}$ mice. Furthermore, cotransfer of regulatory T cells from either WT or CD73$^{-/-}$ mice prevented weight loss and intestinal pathology to an equal degree. Thus, CD4+Treg cells lacking CD73 are not defective in their ability to suppress IBD and therefore are not a major contributor to the severe IBD in CD73$^{-/-}$ mice. From these observations, we determined that, while the lack of CD73 expression on immune cells contributed to the pathogenesis of IBD, this was not the major cause of the extensive pathology and ongoing inflammation observed in CD73$^{-/-}$ mice with colitis. We propose therefore that lack of CD73 expression on the colonic epithelium itself plays a critical role in IBD in CD73$^{-/-}$ mice.

CD73$^{-/-}$ mice also exhibited a higher frequency of CD11c^{+} DCs infiltration in the colon. Mucosal DCs located in the epithelial dome of the colonic patches or in colonic lymphoid follicles are known to be involved in endocytosis and presentation of luminal antigens [42, 43]. The colonic epithelium is critical for maintenance of immune homeostasis in the colonic environment [44–46]. It does this in part by keeping mucosal DCs in a quiescent state [44–46]. The location and increase in these DCs suggest that they may be involved in presentation of luminal antigens and propagation of colonic inflammatory immune responses, driven by the activated inflammatory colonic epithelial cells in CD73$^{-/-}$ mice. It is also possible that CD73 expression on the intestinal vasculature could play a role in regulating intestinal inflammation. Future studies would be able to address these questions.

The intestinal epithelium plays a critical role in maintaining the homeostasis of the intestinal environment [8]. It does this in multiple ways including regulation of TLRs, NF-κB, innate and adaptive immune responses, as well as by providing a physical barrier comprised of junction molecules that separate the luminal contents from the underlying lamina propria [8]. Whole colons from DSS-treated CD73$^{-/-}$ mice produce high levels of TNF-α and IL-1β over the course of DSS treatment. IL-1β was detectable in colonic epithelial preparation in non-DSS-treated CD73$^{-/-}$ mice and remains elevated even after DSS treatment is aborted. Both IL-1β and TNF-α are well-known contributors to the pathogenesis of colitis in mice and humans [32]. Consistent with this ongoing inflammatory program, constitutively high levels of the transcription factor NF-κB, which is a critical mediator in inflammatory and remodeling responses, are also observed. Both IL-1β and TNF-α are potent activators of NF-κB. NF-κB is a key player in the cycle of inflammation in CD and is found constitutively active in colonic epithelial cells in IBD [47]. Adenosine is a negative regulator of NF-κB in human intestinal epithelial cells [36, 47]. The increase in IL-1β levels during and after DSS treatment correlates with active NF-κB protein and ongoing inflammation and pathology in colons of CD73$^{-/-}$ mice. Although IL-1β is observed in non-DSS-treated CD73$^{-/-}$ mice, albeit lower than is seen in DSS treatment, these mice do not display any signs of colon pathology. Therefore, we hypothesize that an inflammatory trigger (DSS) in the absence of extracellular adenosine in CD73$^{-/-}$ mice results in elevated IL-1β that is unresolved even after the trigger is removed. This potentially can contribute to the severe and unresolved inflammation in CD73$^{-/-}$ but not in WT mice.

Intestinal epithelial cells express a spectrum of Toll-like receptors (TLRs), recognize and respond to microbial products (MAMP/PAMPS), and can trigger inflammatory responses by mucosal immune cells. Studies have shown that exposure of the apical side of a polarized epithelial barrier to the TLR9 ligand, CpG DNA, results in a decreased ability to produce inflammatory cytokines, such as IL-6 and IL-8 [41, 48]. However, basolateral stimulation of the epithelial barrier with the same agent resulted in a potent proinflammatory response [48]. Colons of CD73$^{-/-}$ mice with colitis exhibited increased TLR9 expression that is consistent with the heightened and unresolved inflammation in CD73$^{-/-}$ mice but not in WT mice with colitis. Increased TLR9 expression, together with the increase in proinflammatory cytokines such as TNF-α and constitutive NF-κB activity, is indicative of an activated intestinal epithelium. We hypothesize that the ongoing inflammation in CD73$^{-/-}$ mice could potentially be due to a breakdown of the intestinal epithelial barrier that leads to leakage of luminal antigens (PAMPS/MAMPS) into the lamina propria and activation of innate receptors like TLR9. Altered TLR expression is commonly associated with chronic inflammatory diseases including IBD. An inflammatory reaction is only required when an antigen is able to bypass

the protective epithelial barrier. Consistent with the idea of a severely compromised barrier, CD73$^{-/-}$ mice express significantly lower levels of JAM-A protein and decreased levels of JAM-4 and α-catenin transcripts. The increased gastrointestinal permeability in CD73$^{-/-}$ mice would allow easy passage of commensal bacteria or their ligands across the epithelial barrier, resulting in stimulation of basolateral TLRs and production of proinflammatory cytokines. These findings strongly indicate that the lack of CD73 expression on colonic epithelial cells plays a critical role in the ongoing inflammation in CD73$^{-/-}$ mice. This leads us to conclude that extracellular adenosine function in the colon is critical for regulation of colonic immune responses and for the resolution of the inflammatory program and establishment of intestinal homeostasis.

In summary, our data show that the lack of CD73 expression on effector immune cells and on colonic epithelium causes severe IBD in CD73$^{-/-}$ mice. While effector immune cells in CD73$^{-/-}$ mice contribute to IBD pathogenesis, it appears that the greater degree of inflammation and colonic tissue damage is caused by a lack of CD73 expression on the colonic epithelium. This results in breakdown of the epithelial barrier, possibly leading to infiltration of luminal antigens and subsequent presentation of microbial products and activation of immune responses by innate and adaptive immune cells. This perpetuates a vicious cycle of unresolved inflammation due to the lack of CD73-generated adenosine. We conclude therefore that CD73 expression on colonic immune cells is important for immune regulation whereas CD73 expression on colonic tissue is critical for regulating the magnitude and the resolution of the mucosal immune response. These studies provide deeper insights into adenosine's role in mucosal immunity and demonstrate the potential for future development of adenosinergic therapy in treatment of colitis.

Authors' Contribution

A. T. Waickman and D. A. Mohamed contributed equally to this work.

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Review Article

Ectonucleotidases in Tumor Cells and Tumor-Associated Immune Cells: An Overview

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Increasing evidence points out that genetic alteration does not guarantee the development of a tumor and indicates that complex interactions of tumor cells with the microenvironment are fundamental to tumorigenesis. Among the pathological alterations that give tumor cells invasive potential, disruption of inflammatory response and the purinergic signaling are emerging as an important component of cancer progression. Nucleotide/nucleoside receptor-mediated cell communication is orchestrated by ectonucleotidases, which efficiently hydrolyze ATP, ADP, and AMP to adenosine. ATP can act as danger signaling whereas adenosine, acts as a negative feedback mechanism to limit inflammation. Many tumors exhibit alterations in ATP-metabolizing enzymes, which may contribute to the pathological events observed in solid cancer. In this paper, the main changes occurring in the expression and activity of ectonucleotidases in tumor cells as well as in tumor-associated immune cells are discussed. Furthermore, we focus on the understanding of the purinergic signaling primarily as exemplified by research done by the group on gliomas.

1. Introduction

Nucleotide/nucleoside receptor-mediated cell communication is controlled by the action of ectonucleotidases, including the members of the ectonucleoside triphosphate diphosphohydrolases (E-NTPDases, ecto-ATPases, ectoapyrases, EC 3.6.1.5), ectonucleotide pyrophosphatase phosphodiesterases (E-NPP, EC 3.1.4.1), ectoalkaline phosphatases (ALP, EC 3.1.3.1), and ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73, EC 3.1.3.5), which efficiently hydrolyze ATP, ADP and AMP to adenosine (Ado) [1–3].

The E-NTPDase members differ regarding the preferences for nucleotides as substrates. While NTPDase1/CD39 hydrolyses nucleoside tri- and diphosphates almost equally well, NTPDase2/CD39L1 presents a high preference for nucleoside triphosphates and NTPDase3/CD39L3 and 8 reveal an intermediate preference for ATP over ADP [1, 4–9]. In consequence, the action of NTPDase1/CD39 produces almost directly AMP with minor amounts of free ADP in the extracellular space. This functional property implicates the participation of this enzyme in the control of specific P2Y receptors for nucleoside triphosphates. Otherwise, ADP is transiently produced by the action of NTPDase2/CD39L1, which implicates the generation of agonist for nucleoside diphosphate-sensitive receptors such as platelet P2Y1 and P2Y12 receptors [2]. The second family of ectonucleotidases is the ectonucleotide pyrophosphatase/phosphodiesterases (E-NPP). The E-NPP family is constituted by seven ectoenzymes, but only the NPP1–3 are involved in the purinergic signaling [2, 10–12]. The final step of nucleotide hydrolysis to generate adenosine is catalyzed by ecto-5′-nucleotidase/CD73 (ecto-5′-NT/CD73) [1, 13]. In addition, to constitute the major source of extracellular adenosine, other nonenzymatic functions are assigned for this protein.

Ecto-5′-NT/CD73 itself acts as a proliferative factor and is involved in the control of cell growth, cell-cell and cell-matrix interactions [14–16].

In this paper, the alterations in the ATP-metabolizing enzymes, especially the ectonucleotidases that may contribute to the physiopathological events observed in solid cancer are discussed.

2. Ectonucleotidases in Immune Cells

Extracellular nucleotides and nucleosides play an important role in inflammatory and immune responses. To date, ATP is mainly associated to proinflammatory response whereas adenosine has opposite effects limiting the inflammation by suppressing the actions of immune cells [17–20]. Moreover, the plasticity of immune cells during early phase to resolution of inflammation turns important of the control of these immunomodulatory molecules. Increasing evidence suggests the participation of ectonucleotidases in inflammatory process involving immune cells [21–26]. The ectonucleotidases are expressed in B lymphocytes, natural killers cells (NKs), monocytes, macrophages, dendritic cells (DCs) and subsets of T cells [21–26]. Although the presence of the enzymatic chain responsible for ATP hydrolysis and adenosine production was demonstrated in almost all immune cells, only recently the participation of ectonucleotidases in the control of inflammation has been shown.

The first studies to begin to elucidate the physiological role of E-NTPDases (Ecto-ATPases) in immune cells have been proposed in the early nineties [27–29]. Dombrowski et al. [27] showed evidence that Ecto-ATPase activity was required for activation of effector T cells ($CD8^+$) and for antigen recognition [27]. Likewise, upregulation of E-NTPDase activity on $CD4^+$ cells has been described soon after stimulation whereas $CD4^+$ naïve cells present a negligible activity [30]. In the same study it was shown that the inhibition of E-NTPDase or ATP depletion on $CD4^+$ diminished INF-γ and IL-2 secretion [30]. Recently the role of adenosine generated by ecto-5′-nucleotidase/CD73 in graft-versus-host disease was demonstrated. The ecto-5′-nucleotidase/CD73 deficiency led to enhanced T-cell expansion, IFN-γ and IL-6 production, and the migratory capacity of $CD73^{-/-}$ T cells [31].

Recent studies have shown the central role of ectonucleotidases in Foxp3+ T regulatory cells (Tregs). The NTPDase1/CD39 and the ecto-5′-NT/CD73 expressed in Tregs compose one of the immunosuppressive mechanisms associated to these immune cells [32–35]. In addition, alterations in NTPDase1/CD39 and ecto-5′-NT/CD73 machinery may produce more adenosine, which lead to severe immunodeficiency with recurrent infection [36, 37]. In accordance, recently Tang et al. [38] verified that the NTPDase1/CD39 on Foxp3+ T regulatory cells correlates with progression of hepatitis B virus infection and it can be associated with other viral infections, and autoimmune diseases [38]. Moreover, it has been reported that lupus patients express low levels of NTPDase1/CD39, and this is associated with reduced generation of adenosine [39].

In relation to the ectonucleotidases in macrophages, some advances have been done. Hyman et al. [40] have reported the importance of NTPDase1/CD39 in the trafficking of monocyte/macrophage during an ischemic process. They showed that inhibition or genetic deletion of NTPDase1/CD39 ($CD39^{-/-}$) generated an increase in the ischemic area and the leukocytes number (mainly monocytes/macrophages). The data demonstrated that NTPDase1/CD39 reduces stimulation of the P2X7 receptor by modulating $\alpha M\beta 2$ integrin expression on the surface of monocytes/macrophages, thus controlling their migration [40]. Pelegrin and Surprenant [41] have reported the participation of pyrophosphate originated from extracellular ATP hydrolysis to inhibit IL-1β release in alternative/M2 polarized macrophages. In addition, NTPDase1/CD39, the dominant ectonucleotidase on macrophages, controls the IL-1β secretion by these cells by regulating the P2X7 receptor activation [26]. Notably, we have shown that NTPDase1/CD39 and ecto-5′-NT/CD73 are differentially expressed during macrophage polarization, which results in extracellular ATP accumulation in proinflammatory/M1 phenotype while anti-inflammatory or alternative/M2 phenotype generates immunosuppressive adenosine [24].

Although the NTPDase1/CD39 expression has been reported in dendritic cells, little is known about the role of E-NTPDases and other ectonucleotidases in immune function of these cells and its subtypes [42]. For instance, skin-resident dendritic cells (Langerhans cells) in $CD39^{-/-}$ mice reveal a dichotomy role in irritant versus allergic contact dermatitis [43]. In the irritant dermatitis there was an exacerbated skin inflammation in $CD39^{-/-}$ mice indicating that the NTPDase1/CD39 serves as the first line of defense at the environmental interface against nucleotide-mediated inflammatory signals. On the other hand in the allergic contact dermatitis was severely attenuated in these mice by impairing the Langerhans cell with T cell communication in antigen presentation [43].

Corriden et al. [44] showed the participation of NTPDase1/CD39 chemotaxis regulation by facilitating extracellular ATP hydrolysis in human and neutrophil lineage. Corroborating this data, it has been demonstrated that NTPDase1/CD39 controls IL-8 production in human neutrophils via regulation of P2 activation [25]. Even though NTPDase1/CD39 is expressed in NK and NK-T cells, the application of this has not yet been fully elucidated.

Of note, NTPDase1/CD39 and ecto-5′-NT/CD73 expression by tumor-infiltrated immune cells can lead to adenosine generation, inducing an immune suppression around the tumors. So, these ectoenzymes might allow immune cells to adjust the outcome of the extracellular purinergic cascade in order to fine-tune their functions during the inflammatory set. Therefore, the continuing development of therapeutic strategies targeting the combat for the disordered inflammation and aberrant immune reactivity that involve ectonucleotidases could offer promising finding.

3. Ectonucleotidases in Solid Cancer

Cancer development is a multifactorial process consisting of numerous genetic alterations that controls cell proliferation

and differentiation, including the regulation in oncogenes expression (MDM2, CDK4, EGFR) and tumor suppressor genes (p53, p16, p15, and RB1) [45–48]. However, increasing evidence points that the genetic alteration does not guarantee the development of a tumor and indicates that complex interactions of tumor cells with the microenvironment are fundamental to tumorigenesis. In the tumor microenvironment, the presence of secretory products released by tumor and tumor-associated cells creates a growth factor-rich environment linked to tumor maintenance and growth [49]. A number of studies have investigated the identity of these endogenous signals, their receptors, and signaling pathways using tumor models. The most likely candidates are dying cells or extracellular matrix components, glutamate, nucleotides, and nucleosides, for example, ATP and adenosine, all of which were found to be present in the tumor environment [50–52].

Purinergic signaling involving ATP and the respective breakdown or hydrolytic products such as ADP and adenosine activate their own responses via purinergic receptor activation and modulate cross-talk with chemokines [17]. ATP has been identified as a mitogen for v-myc immortalized neural progenitor cells [53]. In astrocytes, extracellular ATP regulates ERK function by activating $P2Y_1$, $P2Y_2$, or $P2Y_4$ purinoceptors [54, 55] indicating the potential for cross-talk with FGF-, EGF- and PDGF- driven cell mitogenic pathways. Adenosine may accumulate in the tumor interstitium [52] where it modulates cell proliferation and angiogenesis, and suppresses anticancer immune responses [56, 57]. Purines can be released from damaged cells during tumor growing, acting as a classical danger signal for the immune system and as a proliferative stimulus to different cancer kinds. However, purines are also released from host normal cells, immune as well as cancer cells through several active mechanisms, including shear stress, hypotonic swelling, hypoxia, stretching, hydrostatic pressure, as well as in response to Ca^{2+}-mobilizing pharmacological agonists [58–60]. As presented before, nucleotide/nucleoside receptor-mediated cell communication is orchestrated by ectonucleotidases, which efficiently hydrolyze ATP, ADP, and AMP to adenosine [61]. The presence/absence of ectonucleotidases in a variety of human tumors has been reported such as ovarian cancer [62], Walker 256 tumor [63], melanomas [64], colorectal cancer [65], glioma [66], and bladder cancer [67]. Therefore, it is tempting to propose that disruption of ectonucleotidase activity from both tumor and infiltrated cells may constitute important regulators of tumor spread and metastasis. Accordingly, it has shown that ATP accumulates in the tumor interstitium at hundreds micromolar range, while being almost undetectable in healthy tissues [51]. Extracellular ATP may be crucial for the tumor not only as a stimulus for growth but also as a source of an immunosuppressive agent such as adenosine [51].

The anti- or protumor effect target by ectonucleotidases, mainly NTPDase1/CD39, is related to tumor kind and its interaction with stromal, immune and endothelial cells. For example, a study published by Häusler et al. [62] showed aberrant NTPDase1/CD39 and ecto-5′-NT/CD73 expression in human ovarian cancer biopsies. Functional assays in ovarian cancer cell culture applying siRNA against NTPDase1/CD39 and ecto-5′-NT/CD73 or pharmacological inhibitors of A_{2A} adenosine receptors revealed that tumor-derived adenosine inhibits the proliferation of allogeneic human $CD4^+$ T cells as well as cytotoxic effect of T cell priming and NK cells cytotoxicity [62]. The presence of E-NTPDase and ecto-5′-NT/CD73 has been characterized in Walker 256 tumor, where the NTPDase1/CD39, NTPDase2/CD39L1, and ecto-5′-NT/CD73 were identified as the dominant enzymes expressed, which by regulating the ratio of nucleotides/nucleosides may target tumor growth [63]. On the other hand, in melanomas, an association was observed between NTPDase1/CD39 overexpression, the differentiation degree of tumor cells, and the tumor escape from immunological effectors mechanisms at early stages of tumor progression, indicating a role of purinergic signaling in cell differentiation and antitumor immune response [64]. Indeed, the deletion of NTPDase1/CD39 resulted in reduction of melanoma growth and inhibition of pulmonary metastases, associated with abrogation of angiogenesis [68]. In addition to ectonucleotidases expressed by tumor cells, the nucleotide-metabolizing enzymes present at surface of tumor-associated cells also contribute to tumor growing or inhibition. The NTPDase1/CD39 expression on Treg inhibits NK cell-mediated antitumor activity and is permissive for hepatic metastatic tumor growth, whereas vascular NTPDase1/CD39 boosts angiogenesis [69]. Extracellular ATP limits melanoma cell growth, and this antitumor effect could be overcome by intrinsic NTPDase1/CD39 expression by endothelial cells [70]. The authors suggest targeting the NTPDase1/CD39 activity or expression in combination with conventional therapy could provide a novel approach to cancer treatment [70]. In human follicular lymphoma, it has been observed that, in addition to Treg-suppressing effect, infiltrating T cells are suppressed by extracellular adenosine, which is produced by ATP-nucleotidase-adenosine system present in lymph node mononuclear cells [71]. Indeed, the selective NTPDase1/CD39 inhibitor and the A_{2A} and A_{2B} antagonists partially overcome T cell suppression [71]. Finally, the increased expression of NTPDase1/CD39 and ecto-5′-NT/CD73 in Treg cells of patients with head and neck cancer is related to the conversion of ATP to immunosuppressive adenosine. Elevations in adenosine levels are responsible for suppressor functions of $CD4^+CD39^+$ Treg in patients with an active disease as well as those with no evident disease after successful therapy [32].

Ecto-5′-NT/CD73, originally defined as a lymphocyte differentiation antigen, is thought to function as a cosignaling molecule on T lymphocytes and is widely expressed on many tumor cell lines and in cancerous tissues [56, 72, 73], including bladder cancer [67], glioma cell lines [74], melanoma [75], ovarian cancer [76], thyroid cancer [77], esophageal cancer [78], prostate cancer [79], breast cancer [80, 81], and lymphoma [82]. Ecto-5′-NT/CD73 upregulation is associated with a highly invasive cancer phenotype, drug resistance, and tumor-promoting functions [56]. In addition, to produce immunosuppressive adenosine from AMP hydrolysis, ecto-5′-NT/CD73 acts as an adhesive molecule and interacts with extracellular matrix glycoprotein, such as

fibronectin and laminin, to produce cancer-invasive properties [56]. Studies suggest that ecto-5′-NT/CD73 expression can enhance breast-cancer cell migration and invasion [81], and its expression has been proposed as prognostic marker to patients. Indeed, the therapy with anti-CD73 monoclonal antibody delayed the breast primary tumor growth and inhibited the development on spontaneous lung metastases [83]. These antitumor effects were dependent on an induction of an adaptive antitumor immune response. In addition, ecto-5′-NT/CD73 was involved in tumor chemotaxis, and the A_{2B} adenosine receptor participates in this process [83]. In line with the role of ecto-5′-NT/CD73 in cancer progression, Zhi et al. [84] evaluated the participation of ecto-5′-NT/CD73 in breast cancer growth by examining the effect of ecto-5′-NT/CD73 suppression via RNA interference and ecto-5′-NT/CD73 overexpression on tumor growth *in vitro* and *in vivo*. As expected, the cell growth rate was significantly lower after ecto-5′-NT/CD73 suppression. In opposite, the ecto-5′-NT/CD73 overexpression increased cell viability and promoted cell cycle progression, depending on its enzyme activity [84]. Taken together, these studies suggest that ecto-5′-NT/CD73 play an important role in cancer growth by affecting cell cycle progression and apoptosis and by triggering adaptive antitumor immunity and inhibiting metastasis [83, 84].

Although the functions of ecto-5′-NT/CD73 in cancer cells have been investigated to some extent, the contribution of host ecto-5′-NT/CD73 activity to cancer progression has been recently addressed. In these studies, authors employed ecto-5′-NT/CD73 gene-targeted mice to investigate the role of host-derived ecto-5′-NT/CD73 in antitumor immunity, tumor cell metastasis, and carcinogenesis [85–87]. Ecto-5′-NT/CD73 deficient mice had significantly elevated ATPase and ADPase activities in T lymphocytes. In a melanoma model, the growth of primary tumors and formation of metastasis were significantly attenuated in mice lacking ecto-5′-NT/CD73. The intratumoral accumulation of Tregs and mannose receptor macrophages, which are related to tumor malignancy, was also attenuated in ecto-5′-NT/CD73-deficient mice [85]. In addition, it has been shown that the host-derived ecto-5′-NT/CD73 ablation significantly suppressed the growth of colon cancer, lymphoma, mammary tumors, and melanoma [86]. The protective effect of ecto-5′-NT/CD73 deficiency on primary tumors was dependent on $CD8^+$ T cells and associated with an increased frequency of antigen-specific $CD8^+$ T cells in peripheral blood and tumors [86]. Finally, recent studies suggest that host-derived ecto-5′-NT/CD73 exerts a critical oncogenic function during tumorigenesis. Ecto-5′-NT/CD73 deficiency suppressed the development of 3-methylcholanthrene- (MCA-) induced fibrosarcomas and also suppressed prostate tumorigenesis in TRAMP transgenic mice. Notably, the treatment with an anti-CD73 monoclonal antibody effectively suppressed growth of established tumors and inhibited the development of TRAMP-C1 lung metastases [87]. Taken together, these data indicate that suppression of ecto-5′-NT/CD73 activity at multiple levels, including tumor cells, Tregs and non-hematopoietic cells, may be a new tool to control tumor growing and modulate antitumor immune responses.

3.1. Ectonucleotidases in a Model of Solid Tumor: Gliomas. Different signaling pathways, including the purinergic system, are involved in glioma progression [66].

It was previously showed that several glioma cells are resistant to cytotoxic ATP while this nucleotide promotes glioma proliferation [88, 89] and neuronal cell death [90]. We have shown that a variety of glioma cell lines (C6, U138MG, U251MG, and U87MG) exhibit diminished ATP hydrolysis (low ATPase/ADPase activities) and elevated capacity of hydrolyze AMP (high AMPase activity) when compared to astrocytes in culture [91]. According to enzymatic activity profile, glioma cells present low expression of NTPDase1/CD39, NTPDase2/CD39L1, and NTPDase3/CD39L3 in relation to astrocytes [66]. The same ectonucleotidases profile can be found in bladder tumor [67].

Notably, we also verified that the coinjection of apyrase (an ATP and ADP scavenger) with C6 glioma cells, in an *in vivo* glioma model, resulted in reduction of tumor growth, which was followed by a decreased inflammatory infiltrate, angiogenesis, and malignant characteristics [66]. NTPDase2/CD39L1 overexpression in C6 glioma cells dramatically increased tumor growth, malignant characteristics, a sizable platelet sequestration and macrophage/microglial activation in the tumor area [92]. The NTPDase2/CD39L1, by preferentially removing ATP, may favor extracellular ADP accumulation and consequent $P2Y_1$ and $P2Y_{12}$ receptor modulation on glioma-associated platelets [1, 2]. These data suggest that the ADP derived from NTPDase2/CD39L1 activity stimulates platelet migration to the tumor area and that NTPDase2/CD39L1, by regulating angiogenesis and inflammation, seems to play an important role in tumor progression [92]. In addition, to promote *in vivo* glioma growth, the NTPDase2/CD39L1 overexpression in tumor cells also modulated systemic inflammatory responses [93].

Likewise, previous studies have shown that C6 glioma cells exhibit NPP1 on the plasma membrane, which are responsible for the hydrolysis of low physiological extracellular ATP concentration (1–10 μM) [94]. Interestingly Aerts et al. [95] have suggested that NPP1 can be a prognostic marker to glioma tumors since high grade tumors (grade II, III and IV) have increased NPP1 expression. Moreover, the ATP accumulation can be an explanation for the induction of NPP1 expression in glioma cells [95], which is in accordance with our hypothesis that ATP being degraded very slowly results in the accumulation of this nucleotide around the tumor [66, 95].

Many studies have demonstrated that the presence of inflammatory infiltrate is involved in tumor progression [96, 97]. In gliomas, the presence of inflammatory infiltrate is directly correlated with tumor malignancy degree [98]. C6 glioma cells, in presence of ATP, release proinflammatory factors, such as MCP-1 and IL-8, important for the recruitment of monocytes and neutrophils, respectively [99]. When these immune cells reach the tumor environment, different stimuli modulate macrophage phenotype [100, 101]. Several studies show that tumor-associated macrophages (TAMs) resemble an anti-inflammatory/M2 phenotype, in contrast to the proinflammatory/M1 phenotype [102, 103]. In agreement with these results, Komohara et al. [96] showed that patients

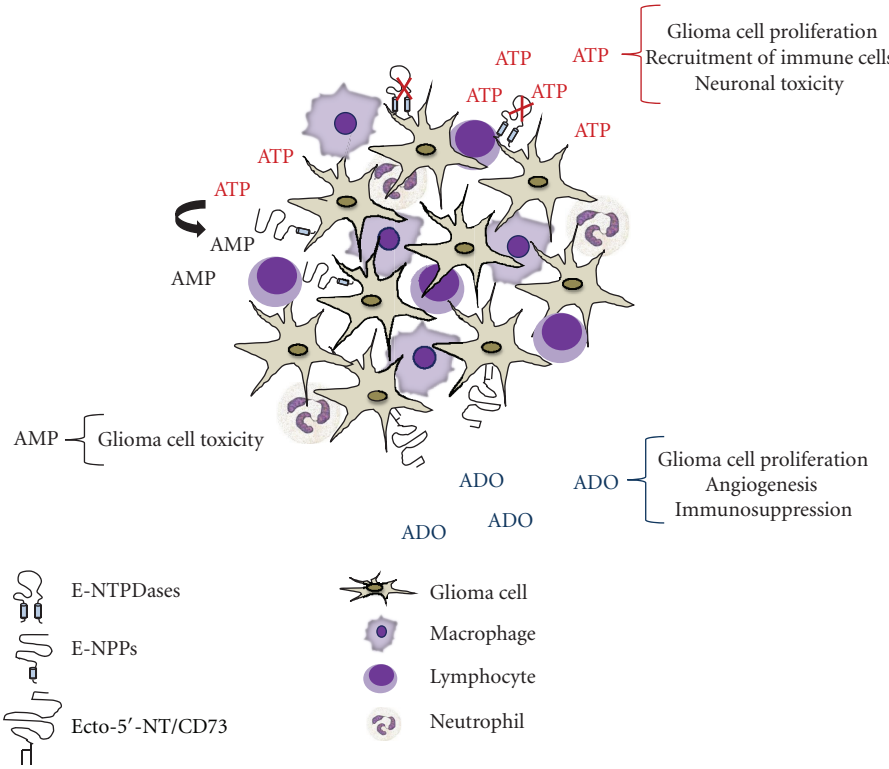

Figure 1: Ectonucleotidases in glioma progression. Glioma cells exhibit low ATP/ADP hydrolysis and a high AMP hydrolysis activity [91]. The inversion of extracellular nucleotide metabolism may favor extracellular ATP and adenosine accumulation within the tumor [51, 52, 66]. ATP could induce neuronal toxicity [90], glioma cell proliferation [88], and recruitment of immune cells by inducing the release of proinflammatory factors by tumor cells, such as MCP-1 and IL-8 [99]. Upon reaching the tumor, different stimuli modulate macrophage to M2 phenotype [100, 101], and studies from our laboratory showed that ectonucleotidases are involved in the differentiation of macrophages [24]. The glioma cells exhibit NPP1 on the plasma membrane [94]; this enzyme generates AMP that is toxic for gliomas [74] but is the substrate for the ecto-5′-NT/CD73 which is highly expressed in glioma [74]. Ado, product of AMP hydrolysis could induce tumor cell proliferation, angiogenesis, and immunosuppression [56]. Therefore, the ATP and its hydrolytic products could be closely related to the immune responses involved in the glioma progression.

with glioblastoma multiforme have an increased infiltration of type M2 macrophages when compared to patients with lower-grade tumors. Therefore, by modulating multiple signaling pathways closely related to tumor malignancy, TAMs are considered key elements in the tumorigenesis processes. Studies are underway in our laboratory to establish if and how the ectonucleotidases would be involved in macrophage polarization in gliomas.

Hydrolysis of AMP by ecto-5′-nucleotidase/CD73 action generates adenosine [1]. Glioblastoma multiforme is characterized by extensive hypoxia areas, which exhibit increased adenosine levels [52]. Adenosine has been recognized to mediate an immunosuppressive response to protect adjacent tissues of inflammation [57]. Furthermore, this nucleoside has been reported as mediator of cell proliferation and angiogenesis and also acts in tumor progression [56].

Previous results from our laboratory showed that increasing confluences led to an increase in ecto-5′-NT/CD73 activity in glioma cell lines [74]. This event could be related to an increased ability to infiltrate the brain parenchyma, which constitutes the main cause of glioma recurrence [104, 105]. It was also shown that the inhibition of this ectoenzyme results in a decreased glioma cell proliferation. We suggested that this process is dependent on adenosine production parallel to AMP removal, a toxic molecule for gliomas [74]. Ohkubo et al. [106] have shown that adenosine inhibits cell proliferation of C6 glioma cells by its intracellular conversion to AMP. However, in our study, we showed that the stimulus of proliferation caused by adenosine is via extracellular effects instead of an adenosine uptake-dependent effect [74].

As in another solid cancer cited herein, the ecto-5′-NT/CD73 is also involved in cell-cell and cell-matrix adhesion, key processes of tumor invasion and metastasis [107]. However, few studies are found in the literature relating the ecto-5′-NT/CD73 in invasion events of gliomas. Gessi et al. [108] showed that adenosine induced an increase of metalloproteinase-9, which is responsible for an increase of glioma cells invasion [108]. In a parallel investigation, we showed that exogenous adenosine promoted an increase in glioma cell adhesion *in vitro*, and the addition of selective inhibitor of this enzyme prevents this effect [109]. Therefore,

this enzyme seems to play extreme importance in the glioma development. Taken together, these data indicate that suppression of ecto-5′-NT/CD73 activity at multiple levels, including tumor cells, may be a new tool to control tumor growing.

4. Concluding Remarks

Nucleotide/nucleoside receptor-mediated cell communication is orchestrated by ectonucleotidases, which efficiently hydrolyze ATP, ADP, and AMP to adenosine. The alterations in ectonucleotidases activity/expression may contribute to the physiopathological events observed in solid cancers as it has been studied in gliomas (Figure 1). In this paper, we summarized the main changes occurring in the expression/activity of ectonucleotidases in glioma cells as well in the tumor-associated immune cells. The development of therapeutic strategies targeting ectonucleotidases in tumor environment could offer promising finding.

Abbreviations

Ado:	Adenosine
ADP:	Adenosine diphosphate
ALP:	Ectoalkaline phosphatase
AMP:	Adenosine monophosphate
Apyrase:	Adenyl-pyrophosphatase
ATP:	Adenosine triphosphate
CDK:	Cyclindependent kinase
DC:	Dendritic cells
Ecto-5-NT/CD73:	Ecto–nucleotidase/CD73
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
E-NPP:	Ectonucleotide pyrophosphatase/phosphodiesterase
E-NTPDase:	Ecto-nucleoside triphosphate diphosphohydrolase
ERK:	Extracellular signal-regulated kinases
FGF:	Fibroblast growth factor
INF-γ:	Interferon-gamma
IL-1β:	Interleukin1 beta
IL-2:	Interleukin 2
IL-6:	Interleukin 6
IL-8:	Interleukin 8
M1:	Classical phenotype/proinflammatory
M2:	Alternative phenotype/antiinflammatory
MCP-1:	Monocyte chemotactic protein-1
MDM2:	Murine double minute 2
NK:	Natural killer cells
PDGF:	Platelet-derived growth factor
RB1:	Retinoblastoma
TAM:	Tumor Associated macrophages
TNF-α:	Tumor necrosis factor alpha
TRAMP:	Trf4/Air2/Mtr4 polyadenylation
Tregs:	T regulatory cells.

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