

Research Article

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

François Ghiringhelli,^{1,2,3} Mélanie Bruchard,^{1,2} Fanny Chalmin,^{1,2} and Cédric Rébé^{1,3}

¹INSERM U866, 21078 Dijon, France

²Faculté de Médecine, Université de Bourgogne, 21079 Dijon, France

³Department of Medical Oncology, Centre Georges François Leclerc, 21000 Dijon, France

Correspondence should be addressed to François Ghiringhelli, fghiringhelli@cgfl.fr and Cédric Rébé, cedricrebe@yahoo.fr

Received 18 May 2012; Accepted 3 July 2012

Academic Editor: Karen M. Dwyer

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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 uracil, 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 phosphatidylinositol 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

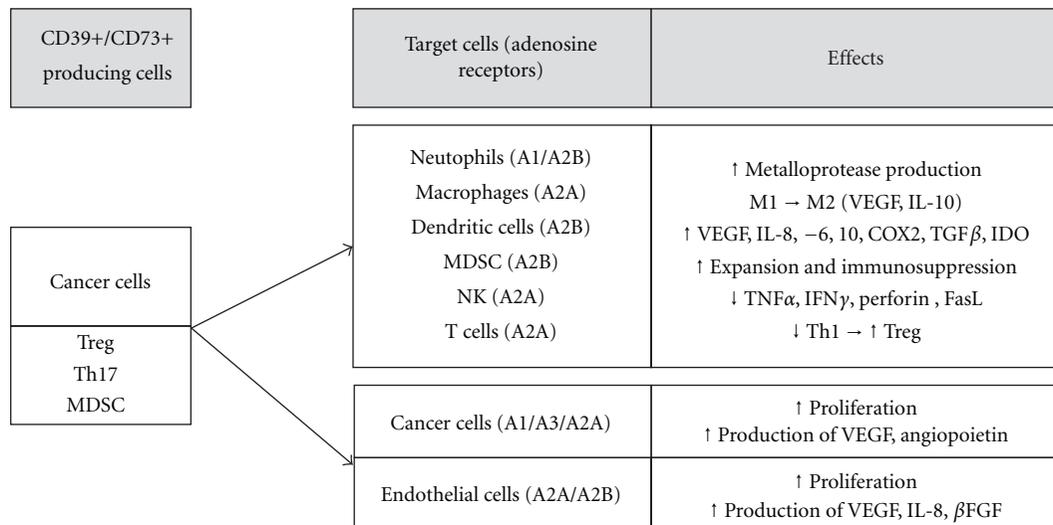


FIGURE 1: Effects of adenosine produced by CD39+/CD73+ cells on target cells. Cancer cells, Tregs, Th17, and MDSCs could produce adenosine through degradation of ATP/ADP by CD39 and CD73. Then adenosine binds on target cells, such as immune cells, cancer cells, or endothelial cells and modifies their 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-1 α (HIF-1 α) 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 β FGF (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

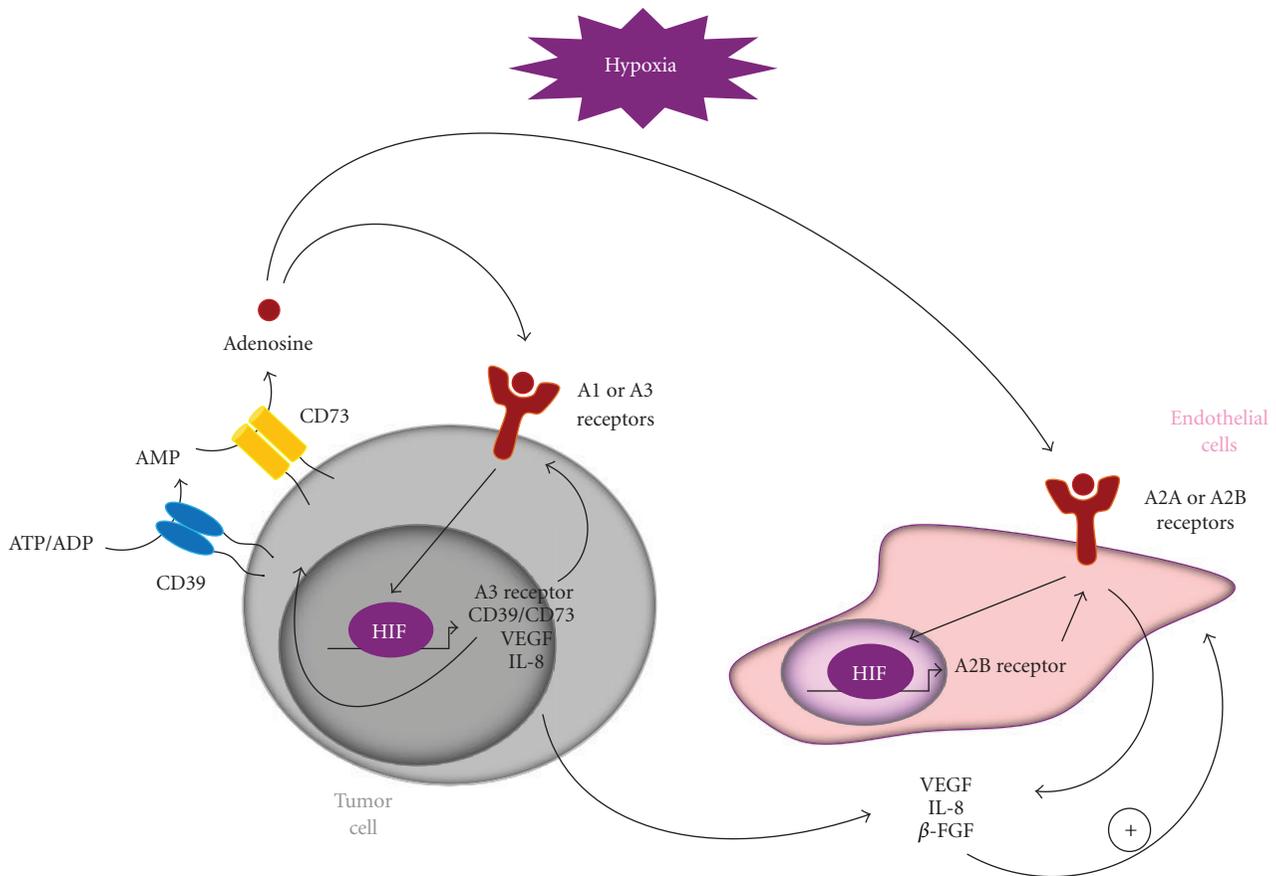


FIGURE 2: Hypoxia mediated expression of ectonucleotidases and adenosine receptors. HIF is induced under hypoxic conditions in cancer cells and directly increases the expression of (1) ectonucleotidases CD39 and CD73, which generate adenosine from ATP/ADP, (2) adenosine receptors that could, after binding 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-1 β [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-1 α 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-1 β , 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-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-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.

Acknowledgments

The authors thank P. Bastable for carefully reading the paper. F. Ghiringhelli was supported by ARC, FRM, INCA, and Ligue contre le Cancer, M. Bruchard by fellowships from the Ministère de l'Enseignement Supérieur et de la Recherche, F. Chalmin by fellowships from Ligue contre le cancer, and C. Rébé by Ligue contre le cancer comité Grand-Est.

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