Increased Ectonucleotidase Expression and Activity in Regulatory T Cells of Patients with Head and Neck Cancer

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Abstract

Purpose: Regulatory T cell (Treg) frequency and activity are increased in cancer patients and play a major role in tumor escape. Although disease progression is favored by the presence of Treg, mechanisms used by Treg to suppress antitumor immunity are unknown. The ectonucleotidases CD39 and CD73 are expressed in Treg and convert ATP into immunosuppressive adenosine. In this study, the involvement of the adenosinergic pathway in Treg-mediated suppression in head and neck squamous cell carcinoma (HNSCC) patients was evaluated.

Experimental Design: HNSCC patients with an active disease ($n = 19$) and patients with no evident disease after therapy ($n = 14$) were studied. Ectonucleotidase expression on $CD4^+$ T cells and $CD4^+CD25^{high}$ Treg was evaluated by flow cytometry and compared with normal controls. Ectonucleotidase activity was also compared within these three groups. The data were analyzed for associations of ectonucleotidase expression/function with disease stage.

Results: The percentages and expression levels of CD39 and CD73 in $CD4^+$ T cells and Treg were greater in HNSCC than in normal controls and highest in patients with no evident disease. Patients' Treg hydrolyzed ATP at higher rates and produced higher levels of adenosine than normal controls' Treg. The increased frequency and enzymatic activity of $CD4^+CD39^+$ cells corresponded to increased adenosine-mediated suppression of effector T cells, which was partly inhibited by ARL67156, an ectonucleotidase inhibitor, and by ZM241385, a selective A_{2a}/A_{2b} receptor antagonist.

Conclusions: $CD39^+$ Treg frequency and adenosine-mediated suppression are significantly increased in HNSCC patients. The adenosinergic pathway is involved in Treg-mediated immunosuppression in cancer and its attenuation could be a promising immunotherapeutic strategy for patients with HNSCC. (Clin Cancer Res 2009;15(20):6348–57)

Head and neck squamous cell carcinoma (HNSCC), arising from the mucosal epithelium, is the fifth most common type of cancer worldwide and the sixth most common cause of cancer-related mortality (1, 2). Although advances in surgical methods, chemotherapy, and radiation have improved patients' treatment and quality of life, the overall outcome and survival rate among patients with this disease have not notably improved (3). Therefore, novel therapeutic strategies are necessary for the treatment of HNSCC. Recent studies have focused on the dynamic interplay and coevolution of antitumor immune responses and tumor progression in HNSCC (4, 5). These studies have shown that T lymphocytes play a major albeit contradictory role in local tumor expansion, metastasis, and neoangiogenesis. In most human cancers, regulatory T cells (Treg), a small subset of $CD4^+$ T cells, are significantly increased in the peripheral blood as well as in the tumor microenvironment (6–8). Treg are phenotypically defined as $CD4^+CD25^{high}FOXP3^+$ and modulate immune responses by suppressing functions of other T cells. To date, at least two types of Treg are known to exist in man: (*a*) naturally occurring Treg, which develop in the thymus and are responsible for maintaining peripheral tolerance using cell contact–dependent or cell contact–independent suppression (9, 10), and (*b*) inducible Treg (Tr1), which arise in the periphery on antigen exposure and

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Translational Relevance

Data in this article highlight the clinical importance of ectonucleotidase activity associated with regulatory T cells (Treg) in the head and neck squamous cell carcinoma. Treg in these patients have increased ectonucleotidase expression (CD39) and produce higher levels of extracellular adenosine, which mediates immunosuppression via A_{2a} receptor expressed on effector T cells. We show that ATP hydrolysis, adenosine production, and suppression of effector T cells by the patients' Treg positively correlate with high CD39 expression on Treg as well as disease activity. Although the clinical significance of the CD39 overexpression in head and neck squamous cell carcinoma patients remains to be confirmed, our data suggest that targeting of the adenosinergic pathway could be a therapeutic option for these patients.

suppress effector T cells mainly by cell contact–independent secretion of cytokines such as interleukin (IL)-10 and transforming growth factor-β (TGF-β; refs. 11, 12). The mechanisms responsible for suppression mediated by Treg in cancer patients are still poorly understood. It is possible that suppression mediated by Treg accumulating in the peripheral blood and/or at the tumor site of patients with cancer facilitate tumor escape from the host immune system and might also be partly responsible for poor responses of these patients to immunotherapy (13).

We have recently shown that the frequency and suppressive function of Treg were persistently elevated in the peripheral blood of HNSCC patients with an active disease as well as patients who were disease-free (with no evident disease) after receiving oncologic therapy (8). These findings suggest that chemotherapy and/or radiation therapy have a significant effect on the phenotype, frequency, and turnover dynamics of Treg. However, the full effect of Treg-mediated effects on disease progression and response to immunotherapy in HNSCC remains unclear.

It has been shown that murine and human Treg participate in the conversion of extracellular ATP and ADP to adenosine (14–16). The dephosphorylation of ATP and ADP to AMP is mediated by surface-associated CD39, whereas CD73 further reduces AMP to adenosine. Initially, Kobie et al. reported that CD73 is overexpressed on Treg (16), and based on the observations of Ohta and Sitkovsky (17), these investigators connected the ectoenzyme expression to downstream signaling via the A_{2a} receptor ($A_{2a}R$) on effector cells. Adenosine suppresses functions of immune effector T cells by binding to $A_{2a}R$ expressed on their cell surface (17–20). Subsequently, Robson's group identified CD39 overexpression in Treg, which functions upstream of CD73 (14), and extended previous studies to establish the *in vivo* role of $A_{2a}R$ in immunosuppression. In aggregate, these earlier studies have established that Treg can mediate immunosuppression by contributing to the ATP breakdown and adenosine generation.

Here, we show that the frequency of $CD4^+CD39^+$ T cells in the periphery and their enzymatic activity (hydrolysis of ATP) are increased in HNSCC; therefore, extracellular adenosine production is higher in these patients relative to normal controls. Elevations in adenosine levels are responsible for suppressor functions of single-cell–sorted $CD4^+CD39^+$ Treg in patients with an active disease as well as those with no evident disease after successful therapy. Further, HNSCC patients with a late-stage disease have a significantly higher frequency and activity levels of adenosine-generating $CD4^+CD39^+$ Treg than those with an early-stage disease.

Materials and Methods

HNSCC patients and healthy volunteers. Peripheral venous blood samples were obtained from 33 HNSCC patients and 15 age-matched normal controls. All patients were seen in the Outpatient Clinic of the Department of Otolaryngology at the University of Pittsburgh Medical Center between November 2007 and July 2008. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh. The normal controls cohort included 4 females and 11 males with a mean age of 57 ± 12 years (range, 45-67 years). The patient cohort included 8 females and 25 males with a mean age of 62 ± 12 years (range, 41-77 years). The clinicopathologic data for the patient group are listed in Table 1. At the time of blood draws, 19 patients had an active disease, including 18 newly diagnosed cases and 1 patient with a recurrent disease. The other 14 patients had no evidence of disease following oncologic therapies. In all of the patients, a surgical removal of the primary tumor was done and 7 patients additionally received an adjuvant radiochemotherapy. When applied, radiochemotherapy was finished from 3 weeks to 12 months before the time of blood draws for immunologic studies.

Collection of peripheral blood mononuclear cells. Blood samples (20-30 mL) were drawn into heparinized tubes and centrifuged on Ficoll-Hypaque gradients (GE Healthcare Bioscience). Peripheral blood mononuclear cells (PBMC) were recovered, washed in AIM-V medium (Invitrogen), counted in a trypan blue dye, and immediately used for experiments.

Separation of Treg. $CD4^+CD25^-$ T cells, which served as responder cells, were freshly isolated by negative selection from PBMC (normal controls and HNSCC) using the Regulatory T cell Separation Kit and AutoMACS (Miltenyi Biotech). $CD4^+CD39^+$ and $CD4^+CD25^{high}$ Treg cells were single-cell–sorted from PBMC following staining for lymphocytes with the relevant markers.

Antibodies. The following anti-human monoclonal antibodies (mAb) were used for flow cytometry: anti-CD3-ECD, anti-CD4-ECD, anti-CD4-PC5, anti-CD25-PC5, anti-FOXP3-FITC (clone PCH101), anti-CD39-FITC, anti-CD39-PE, anti-CD73-PE, unconjugated anti-CD73, and anti-CTLA4-PE. Antibodies and their respective isotypes, which served as negative controls for surface as well as intracellular staining, were purchased from Beckman Coulter, except for anti-FOXP3 (clone PCH101), anti-CD39-FITC, and anti-CD39-PE, which were purchased from eBioscience. Also, anti-CD73-PE was purchased from BD Pharmingen. The anti-CTLA4-PE antibody was purchased from R&D Systems, and the unconjugated anti-CD73 antibody was purchased from Santa Cruz Biotechnology. The FITC-conjugated AffiniPure goat anti-mouse secondary antibody was purchased from Jackson ImmunoResearch. Before use, all antibodies were titrated using activated as well as nonactivated PBMC to determine the optimal staining dilution for each.

Surface and intracellular staining. Freshly isolated cells were stained for flow cytometry as described previously (1). Briefly, cells were incubated with the antibodies for surface markers for 30 min at 4°C in the dark and then fixed with 2% (w/v) paraformaldehyde in PBS for 15 min. Afterwards, the cells were permeabilized with 0.1% (w/v) saponin in PBS for 30 min and stained with antibodies specific for intracellular markers for 30 min at 4°C in the dark. Cells were washed twice with 0.1% saponin in PBS, resuspended in a flow solution, and immediately analyzed by flow cytometry. Appropriate isotype controls were included for each sample.

Table 1. Clinicopathologic characteristics of enrolled HNSCC patients

Age (y), range	41-77
Sex	
Male	25
Female	8
Tumor site	
Nasopharynx	2
Oral cavity	13
Oropharynx	3
Larynx	12
Not determined	3
Tumor differentiation	
G_1	8
G_2	15
G_3	2
G_x	8
Tumor stage	
T_1	8
T_2	8
T_3	2
T_4	4
T_x	11
Nodal status	
N_0	14
N_1	0
N_2	8
N_3	1
N_x	10
Status at blood draw	
Active disease	
Primary disease	18
Recurrent disease	1
No evident disease	
Primary disease	12
Recurrent disease	2
Disease stage (active disease)	
Early stage	10
Advanced stage	9
Therapy before blood draw (no evident disease)	
Surgery alone	7
Surgery + radiochemotherapy	7

Flow cytometry. Flow cytometry was done using a EPICS XL-MCL flow cytometer equipped with Expo32 software (Beckman Coulter). The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward scatter and side scatter. Forward scatter and side scatter were set in a linear scale, and at least 10^5 cells were acquired for analysis, which was done using the Coulter EXPO 32vl.2 analysis program. For additional analyses, gates were restricted to the $CD3^+CD4^+$, $CD3^+CD4^+CD39^+$, $CD3^+CD4^+CD73^+$, $CD4^+CD25^{high}$, or $CD4^+FOXP3^+$ T-cell subsets.

ATP hydrolysis assay. Magnetic cell-sorted $CD4^+CD25^-$ or $CD4^+CD25^{high}$ cells (25×10^3 per well) from the normal controls or HNSCC patients were incubated in wells of flat-bottomed 96-well plates for 30 min with 10 μmol/L exogenous ATP (Sigma-Aldrich). Some wells were preincubated with ARL67156, an ectonucleotidase inhibitor (Sigma Aldrich), for 30 min before the addition of exogenous ATP at the final concentration of 250 μmol/L. The concentration of "unhydrolyzed" ATP was determined by measuring the frequency of luminescent events (CPM) in a luciferase-based detection system (ATP Lite Luminescence ATP Detection Assay System purchased from Perkin-Elmer). The plates were examined in a Microplate Scintillation and Luminescence Counter (Packard). The average count was determined from triplicate wells. The percentage of ATP hydrolysis was determined by using the following formula: $[1 - (\text{CPM ATP}_{\text{sample}})/(\text{CPM ATP}_{\text{alone}} + \text{CPM ATP for cells}_{\text{alone}})] \times 100$.

Mass spectrometric analysis of adenosine production. $CD4^+CD25^-$ or $CD4^+CD25^{high}$ T cells (25,000 per well) from normal controls as well as HNSCC patients were incubated with 10 μmol/L exogenous ATP in wells of 96-well flat-bottomed plates. To selected wells, ARL67156 or α,β-methylene ADP, an ecto-5′-NT/CD73 inhibitor (100 μmol/L; Sigma-Aldrich), was added. Cell supernatants were collected after various incubation time points. Samples were centrifuged and boiled for 2 min and stored on dry ice until analysis. Adenosine was measured on a ThermoFinnigan LCQ Duo mass spectrometer equipped with electrospray ionization. The samples were separated using a C18 column (Eclipse XDB-C18, 4.6 × 150 mm, 5 μm). A mobile phase consisted of 0.1% formic acid water and methanol solution. The flow rate of a mobile phase was 0.6 mL/min. The analytes were monitored with single-ion monitoring in the positive-ion mode; for adenosine, the mass-to-charge ratio was 268; for 10-13C-adenosine (internal standard), the mass-to-charge ratio was 278. The internal standard (10-13C-adenosine) in supernatants is 10 pg/μL. The average concentration of adenosine was determined in duplicate wells.

Suppression assays. Single-cell–sorted $CD4^+CD39^+$ cells were tested for suppression of proliferation activity in cocultures with autologous $CD4^+CD25^-$ responder cells as described previously (21). CFSE-labeled autologous $CD4^+CD25^-$ cells (10^5 per well) were incubated in wells of flat-bottomed 96-well plates at the suppressor/responder cell ratios of 1:1, 1:2, 1:5, and 1:10. Using the same assay format, either ARL67165 (250 μmol/L) or MRS1191 (0.5 μmol/L; both from Sigma-Aldrich); ZM241385 (0.3 μmol/L), MRS1706 (0.5 μmol/L), or DPCPX (0.5 μmol/L; all from Tocris Bioscience); or neutralizing anti-TGF-β1 mAb and/or anti-IL-10 mAb (1 μg/mL; both from R&D Systems) or appropriate isotype controls were added to selected wells 30 min before adding the suppressor cells. To induce proliferation, responder cells were stimulated with plate-bound OKT-3 (2 μg/mL) and soluble anti-CD28 mAb (2 μg/mL; Miltenyi) in the presence of 150 IU/mL IL-2 for 5 days. All CFSE data were analyzed using the ModFit software provided by Verity Software House as described previously (21).

Immunofluorescence. HNSCC tissue samples were embedded in OCT, and 5 mm frozen sections were cut in a cryostat, fixed for 10 min in cold acetone/ethanol (1:1), and dried at room temperature. The following anti-human antibodies were used for staining: anti-CD4-FITC, CD25-PE (BD Pharmingen), anti-CD39, and anti-CD73 (Santa Cruz Biotechnology). As secondary antibody, Cy5-labeled donkey anti-rabbit (Jackson ImmunoResearch) was used. To eliminate nonspecific binding of secondary antibodies, tissue sections were incubated with 10% donkey serum for 1 h and then washed extensively in PBS. Sections were incubated with the primary antibodies for 1 h at room temperature in a moist chamber. Next, slides were washed and incubated with the secondary antibodies under the same conditions. Primary antibodies were omitted in all negative controls. Sections were mounted in a mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories) to trace cell nuclei. Slides were evaluated in the Olympus Provis (Olympus) fluorescence microscope under ×400 magnification. For digital image analysis, the software Adobe Photoshop 6.0 version was used.

Statistical analysis. All data are mean ± 1 SD of at least three experiments and medians. Data were analyzed using the Kruskal-Wallis test or Wilcoxon Mann-Whitney test for comparisons across outcome groups (normal controls versus patients with an active disease versus patients with no evident disease). Data are presented in box plots showing the mean, median, and interquartile range. Changes in adenosine production and ATP hydrolysis were analyzed with the Student's *t* test. Analyses were done using SAS version 9.1 or Stat Xact 8 and a significance level of 0.05 was assumed.

Results

Expression of CD39 on $CD4^+$ T cells and Treg in the peripheral blood of HNSCC and normal controls. PBMC were obtained from normal controls and HNSCC patients who either had

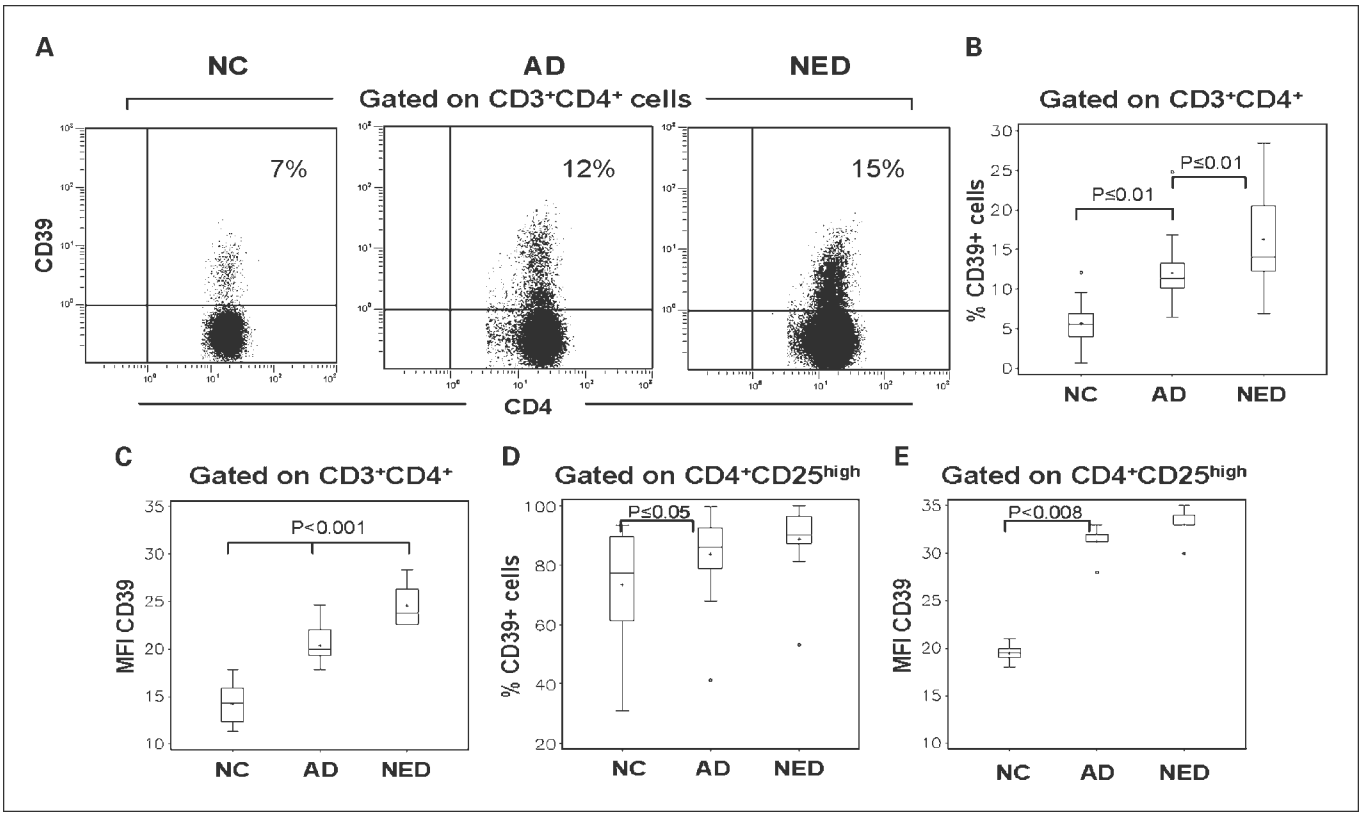

Fig. 1. Increased CD39 expression in $CD3^+CD4^+$ and $CD4^+CD25^{high}$ T cells in patients with HNSCC. *A*, percentages of $CD39^+$ cells within the $CD4^+$ T-cell population in the peripheral blood of a representative normal controls and two HNSCC patients, one with an active disease and one with no evident disease. *B*, percentages of $CD39^+$ T cells in the $CD4^+$ T-cell population of normal controls and all patients were determined by flow cytometry. Data from at least 14 individual experiments per group. *C*, MFI for CD39 on $CD4^+$ T cells in all patients and normal controls was determined by flow cytometry. Percentages of CD39 expression (*D*) and levels of expression (*E*) of CD39 in $CD4^+CD25^{high}$ cells (cells with MFI > 120 for CD25 expression) were determined by flow cytometry for at least 14 individual experiments per group.

an active disease or had no evident disease. The frequency of $CD4^+CD39^+$ T cells was initially examined by flow cytometry in the total $CD3^+CD4^+$ cell population in PBMC. Patients with an active disease had significantly higher frequency of $CD4^+$ $CD39^+$ T cells than normal controls [12 ± 4% (median, 11.35) versus 6 ± 4% (median, 5.5); $P < 0.01$; Fig. 1A and B]. Also, the level of expression, the mean fluorescence intensity (MFI), of CD39 on $CD4^+$ cells was increased in patients with an active disease versus normal controls [20 ± 4% (median, 20.0) versus 14 ± 4% (median, 14.3); $P < 0.001$; Fig. 1C]. $CD4^+$ T cells of patients with no evident disease expressed even higher levels of CD39 [MFI = 24 ± 4 (median, 23.8) versus 20 ± 4 (median, 20.0); $P < 0.001$; Fig. 1C] and these patients also had the highest frequency of $CD4^+CD39^+$ cells [15 ± 8% (median, 14.0) versus 12 ± 4% (median, 11.35); $P < 0.01$; Fig. 1A and B].

Because $CD25^{high}$ and FOXP3 have been generally used as markers for human Treg, we have examined whether $CD4^+CD25^{high}$ cells, of which 83 ± 10% were also $FOXP3^+$, as reported previously by us (ref. 8; range, 67-95%; $n = 15$), expressed CD39. Gating on $CD4^+CD25^{high}$ cells (MFI > 120; ref. 8), we observed higher expression levels and frequencies of CD39 compared with $CD4^+CD25^-$ cells in all three cohorts (data not shown). Further, the frequency and MFI of $CD4^+CD25^{high}$ $CD39^+$ were significantly higher in the peripheral blood of HNSCC than in normal controls, with the highest percentage and levels of CD39 expression observed in the peripheral blood of patients with no evident disease (Fig. 1D and E). Gating on $CD4^+CD39^+$ cells, we observed that up to 80% of these cells were $FOXP3^+$ and mostly CTLA-4^+ in all three cohorts (data not shown).

Expression of CD73 in $CD3^+CD4^+$ T cells and Treg in the peripheral blood of HNSCC and normal controls. The final step in adenosine production is catalyzed by CD73, a 5′-ectonucleotidase. Because adenosine is the metabolite responsible for immunosuppression of effector T cells, it was important to determine expression and activity of CD73 in $CD4^+$ T cells and Treg. Similar to our previously reported data, intracellular staining for CD73 revealed that 75 ± 6% of $CD4^+CD25^{high}$ are $CD73^+$ compared with 15 ± 4% of non-Treg.[7] In $CD3^+CD4^+$ T cells, the level of CD73 expression was increased in patients with an active disease compared with normal controls (data not shown). In Treg, the frequency as well as MFI of CD73 were increased in the peripheral blood of patients with HNSCC compared with normal controls, although the difference was not statistically significant (data not shown). Most of the $CD4^+CD73^+$ cells coexpressed $FOXP3^+$ and $CTLA4^+$ in all three cohorts (data not shown).

[7] M. Mandapathil, B. Hilldorfer, M.J. Szczepanski, M. Czystowska, M. Szajnik, J. Ren, S. Lang, E.K. Jackson, E. Gorelik, T.L. Whiteside. Generation and accumulation of immunosuppressive adenosine by human $CD4^+CD25^{high}$ $FOXP3^+$ regulatory T cells (Treg). Article under review.

Correlation of CD39 expression with disease progression and radiochemotherapy. To determine whether any relationship could be established between percentages of $CD39^+$ cells present in the peripheral circulation and disease progression, we divided HNSCC patients with an active disease into those with an early-stage disease without lymph node metastasis and those with an advanced-stage disease and involved lymph nodes (Table 1). The frequency of $CD4^+CD39^+$ T cells was significantly increased ($P < 0.01$) in patients with the late-stage disease compared with those with the early-stage disease [8 ± 5% (median, 8.15) versus 11 ± 4% (median, 90.5); Fig. 2A]. A significant increase in the level of CD39 expression (MFI) was also observed in the late-stage disease group ($P < 0.01$; data not shown). The percentage of $CD39^+$ Treg within the $CD4^+CD25^{high}$ subset was as well significantly higher in patients with the late-stage disease [82 ± 11% (median, 80.7) versus 89 ± 6% (median, 90.5); $P < 0.03$; Fig. 2B].

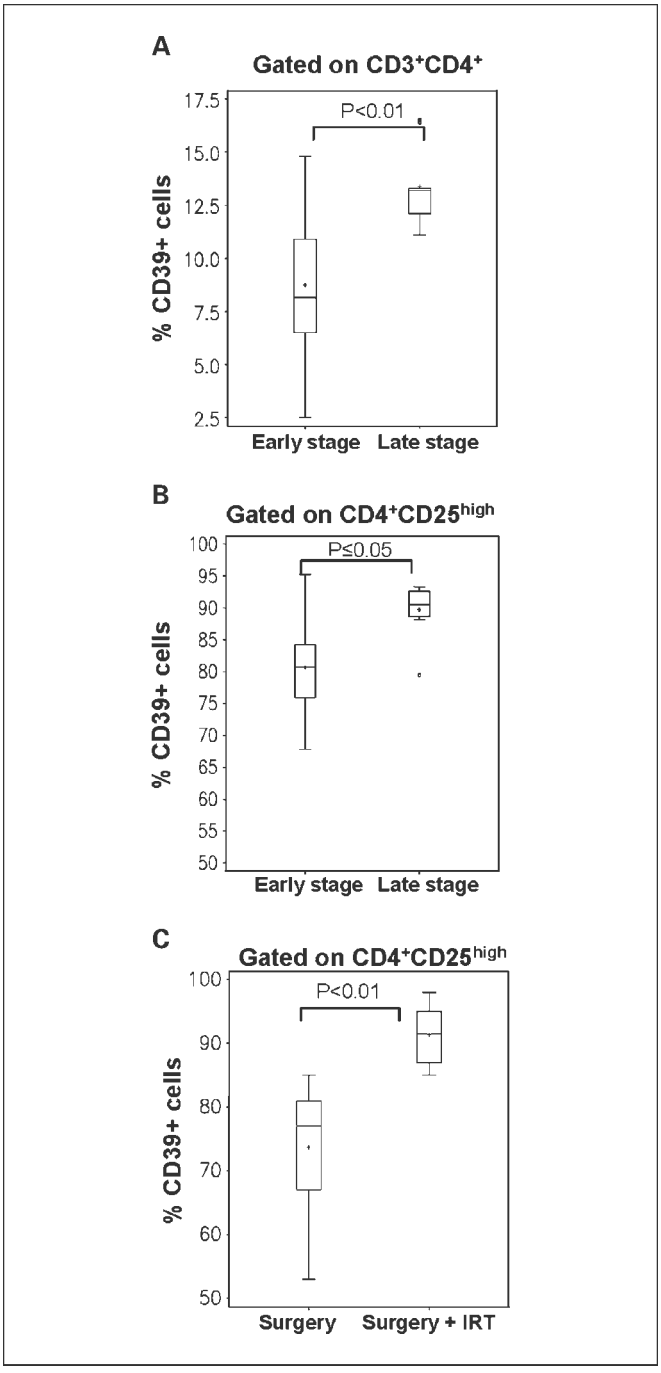

Fig. 2. Correlations of ectonucleotidase expression on T-cell subsets with disease progression and surgery versus surgery + radiochemotherapy. Patients with an active disease were subdivided into (*a*) patients with an early-stage disease and (*b*) patients with an advanced-stage disease. Percentages and levels of CD39 expression are shown. Mean ± SD. The frequency of CD39 expression on $CD3^+CD4^+$ cells (*A*) and $CD4^+CD25^{high}$ cells (*B*) was determined in patients with an active disease, whereas early-stage disease patients ($n = 10$) were compared with patients with an advanced-stage disease ($n = 9$). *C*, patients with no evident disease were subdivided into (*a*) patients treated with surgery alone ($n = 7$) and (*b*) patients treated with surgery and combined radiochemotherapy ($n = 7$). The frequency of CD39 expression was determined on $CD4^+CD25^{high}$ Treg in both groups.

Among patients with no evident disease, a significant increase in the percentage of $CD39^+$ Treg was observed in patients who were treated with surgery in combination with radiochemotherapy compared with patients treated with surgery alone [83 ± 6% (median, 77.0) versus 92 ± 4% (median, 91.5); $P < 0.01$; Fig. 2C]. Overall, these phenotypic data suggest that significant changes in the frequency of $CD4^+CD39^+$ and $CD4^+CD25^{high}CD39^+$ cells occur with disease progression and that therapy has an effect on the frequency of $CD4^+CD39^+$ T cells in the peripheral circulation.

In situ ***analysis of $CD39^+$ and $CD73^+$ Treg in head and neck tumor tissues.*** Next, the presence and distribution of $CD4^+CD39^+$ Treg in HNSCC biopsy tissue was evaluated using multicolor immunofluorescence and confocal microscopy (Fig. 3A-D). Tumor tissue sections were stained with labeled mAb. As shown in Fig. 3C, $CD4^+CD25^+CD39^+$ cells (*violet*) infiltrate the tumor tissue. In fact, most of the $CD4^+CD25^+$ cells (*yellow*) in the tumor express CD39 and are localized in the proximity of $CD4^+CD25^-$ cells (*green*; see *inset*), suggesting that these cell subsets interact *in situ*. However, Treg are not the only cells in the tumor that express CD39. Endothelial cells have been reported to also express CD39 and show increased activity in the tumor microenvironment facilitating neoangiogenesis (22). As shown in Fig. 3D, most of the $CD73^+$ cells are colocalized to $CD4^+CD25^+$ cells (*violet*). These cells seem to be arranged in clusters, which are localized close to effector T cells. Also, the tumor cells seem to express CD73. These findings support the conclusion that $CD39^+$ Treg as well as $CD73^+$ Treg accumulate in HNSCC tissues and are in a direct contact with effector T cells.

Hydrolysis of exogenous ATP by Treg in HNSCC versus normal controls. Although the frequency and expression levels for the ectonucleotidases CD39 and CD73 on Treg were higher in HNSCC patients than normal controls, it was important to determine whether their enzymatic activities were also increased in parallel. Single-cell–sorted $CD4^+CD25^{high}$ cells were, therefore, obtained from the peripheral blood of subjects in all three cohorts and incubated with 10 μmol/L exogenous ATP for 60 min. Compared with normal controls, Treg of patients with an active disease or no evident disease hydrolyzed significantly more ATP ($P < 0.05$; Fig. 4A). On the prior addition of ARL67156, a selective inhibitor of ecto-ATPases, the ability of Treg cells to hydrolyze ATP was significantly decreased in all three groups ($P < 0.001$; Fig. 4A). Subdivision of the patients with an active disease into early and advanced stages revealed an increase in Treg-mediated hydrolysis of ATP in cells obtained from patients with an advanced-stage disease (data not shown).

Adenosine production by Treg in HNSCC versus normal control cells. To further analyze the activity of the ectonucleotidases

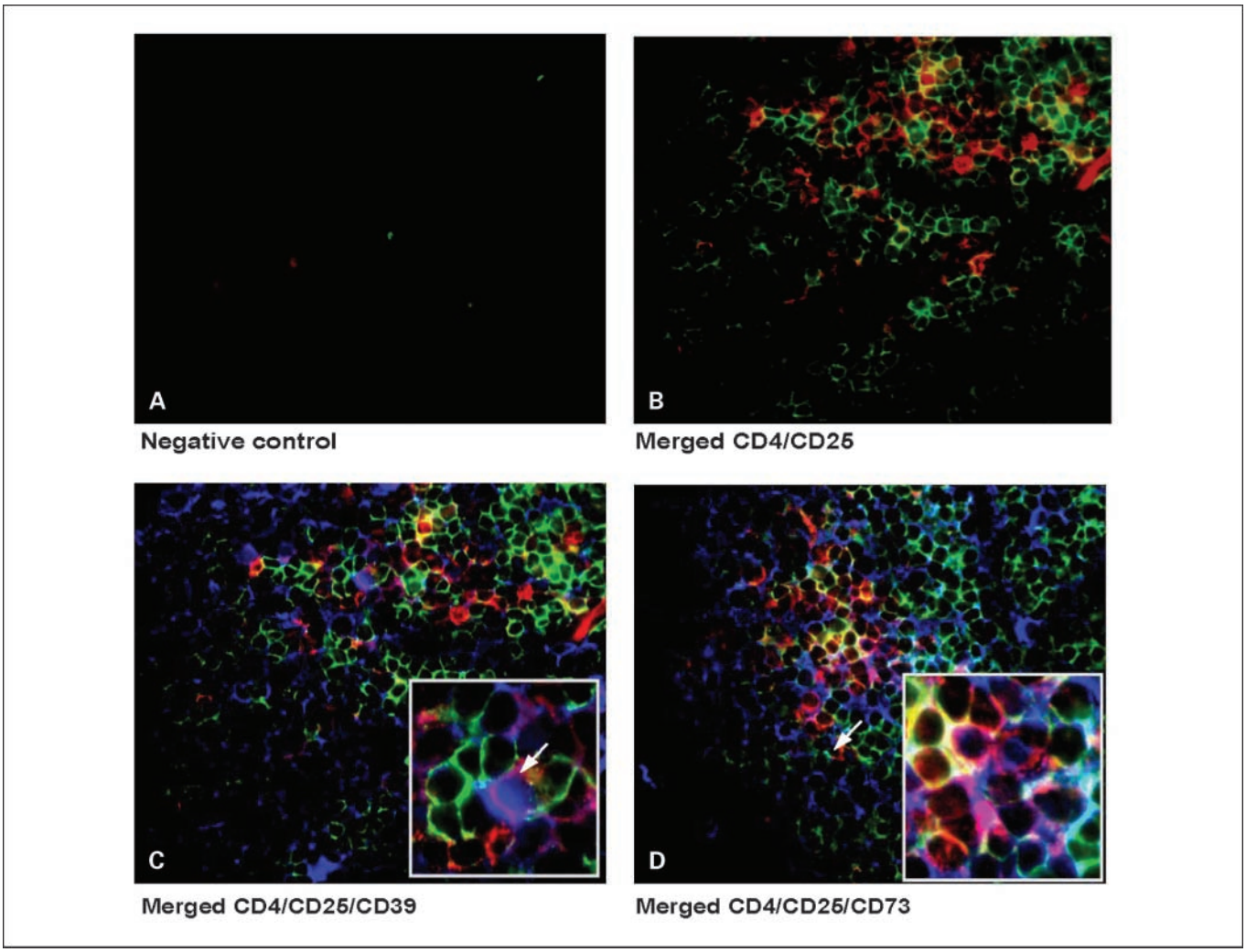

Fig. 3. CD39 and CD73 expression on T cells in HNSCC tissues. $CD4^+CD25^+$, $CD4^+CD25^+CD39^+$, and $CD4^+CD25^+CD73^+$ T cells are shown in sections of a representative tumor of five examined. Magnification, ×400 (*A-D*) and ×600 (*C* and *D, inset*). *A,* negative control. *B, green,* $CD4^+$ cells; *red,* $CD25^+$ cells; *yellow,* $CD4^+CD25^+$ cells. *C,* section stained for CD4, CD25, and CD39: *blue,* $CD39^+$ cells; *violet,* $CD4^+CD25^+CD39^+$ cells; *red,* $CD4^+CD25^-CD39^-$ T cells. *D,* section stained for CD4, CD25, and CD73: *red,* $CD4^+$ cells; *green,* $CD25^+$ cells; *blue,* $CD73^+$ cells. Localization of $CD4^+CD25^+CD73^+$ Treg (*violet*) can be observed in the proximity of $CD4^+CD25^-$ cells (*red*).

CD39 and CD73 in Treg, we compared the ability of $CD4^+CD25^{high}$ Treg and autologous $CD4^+CD25^-$ cells obtained from patients with an active disease to produce adenosine following the addition of 10 μmol/L exogenous ATP. Adenosine levels were measured in the cell supernatants collected at various time points after ATP addition. $CD4^+CD25^{high}$ cells produced more adenosine than $CD4^+CD25^-$ cells (Supplementary Table S1; $P \leq 0.001$). Compared with Treg of normal controls, those isolated from patients with an active disease produced almost six times more adenosine after 60 min (Table 2). Also, when no exogenous ATP was added to the cultures, the amount of adenosine after 60 min measured in the supernatant of Treg from patients with an active disease was significantly increased compared with that measured with Treg of normal controls (Table 2; $P \leq 0.001$). On coincubation of $CD4^+CD25^{high}$ cells with ARL67156, adenosine production was almost completely blocked (Table 2; $P < 0.001$). Also, we observed a complete inhibition of adenosine production by $CD4^+CD25^{high}$ cells when α,β-methylene ADP was added to selected wells (Table 2; $P \leq 0.001$).

Suppressor function of circulating $CD4^+CD39^+$ in HNSCC versus normal controls. To analyze suppressor activity of $CD4^+CD39^+$ cells, single-cell–sorted $CD4^+CD39^+$ cells suppressor cells from freshly isolated PBMC were coincubated with autologous $CD4^+CD25^-$ responder cells at different suppressor/responder cell ratios. After a 5-day culture, the mean suppressor activity of $CD4^+CD39^+$ cells at the 1:1 suppressor/responder cell ratio was 38 ± 3% in normal controls, whereas an increase up to 82 ± 3% in patients with an active disease and up to 93 ± 5% in patients with no evident disease were observed (Fig. 4B). The suppression of proliferation linearly decreased on further dilution of suppressor cells (Fig. 4B). Because Treg from HNSCC patients are increased in ectonucleotidase expression and activity, the generation of high levels of adenosine most likely contributes to the suppressive function of these cells.

Effects of ectonucleotidase inhibitors on suppression mediated by $CD4^+CD39^+$ cells. To further test the hypothesis that adenosine generated by Treg due to ectonucleotidase activity is responsible

for immunosuppression of responder cell proliferation, additional coculture experiments were done. Specifically, the addition of ARL67156, a structural analogue of ATP and an ectonucleotidase inhibitor, to cocultures of $CD4^+CD39^+$ cells serving as suppressor cells with autologous responder cells resulted in a significant decrease of the suppression compared with cultures without the inhibitor in patients with an active disease ($82 \pm 9\%$ versus $22 \pm 1\%$; $P < 0.001$; Fig. 4C) and patients with no evident disease ($93 \pm 5\%$ versus $23 \pm 4\%$; $P < 0.001$; Fig. 4D).

Effects of adenosine receptor antagonists on suppression mediated by $CD4^+CD39^+$ cells. Because the immunosuppressive effects of adenosine are known to be mediated via the $A_{2A}R$, which is expressed on effector T cells (23), we expected to see a decrease of suppression in responder cell proliferation, when this receptor was blocked on effector T cells. Therefore, we added ZM241385, a selective A_{2a} and A_{2b} receptor antagonist, to the above described responder/suppressor cell cocultures. As expected, the addition of ZM241385 significantly blocked the suppression mediated by $CD4^+CD39^+$ cells at the 1:1 suppressor/responder cell ratio in patients with an active disease ($20 \pm 4\%$ versus $82 \pm 9\%$; $P < 0.001$) and patients with no evident disease ($25 \pm 3\%$ versus $92 \pm 4\%$; $P < 0.001$; as shown in Fig. 4C and D). An almost complete block of $CD4^+CD39^+$ Treg-mediated immune suppression was observed after adding ZM241385 and neutralizing anti-TGF-β and anti-IL-10 mAbs to the cocultures ($9 \pm 2\%$ versus $83 \pm 3\%$; $P < 0.001$; Fig. 4E). Addition of other adenosine receptor antagonists such as DPCPX (a selective A_1 receptor antagonist), MRS1191 (a selective A_3 receptor antagonist), or MRS1706 (a selective A_{2b} receptor antagonist) did not show any effect on $CD39^+$ T cell–mediated suppression on responder cell proliferation. In aggregate, the data suggest that suppression of effector T-cell proliferation by

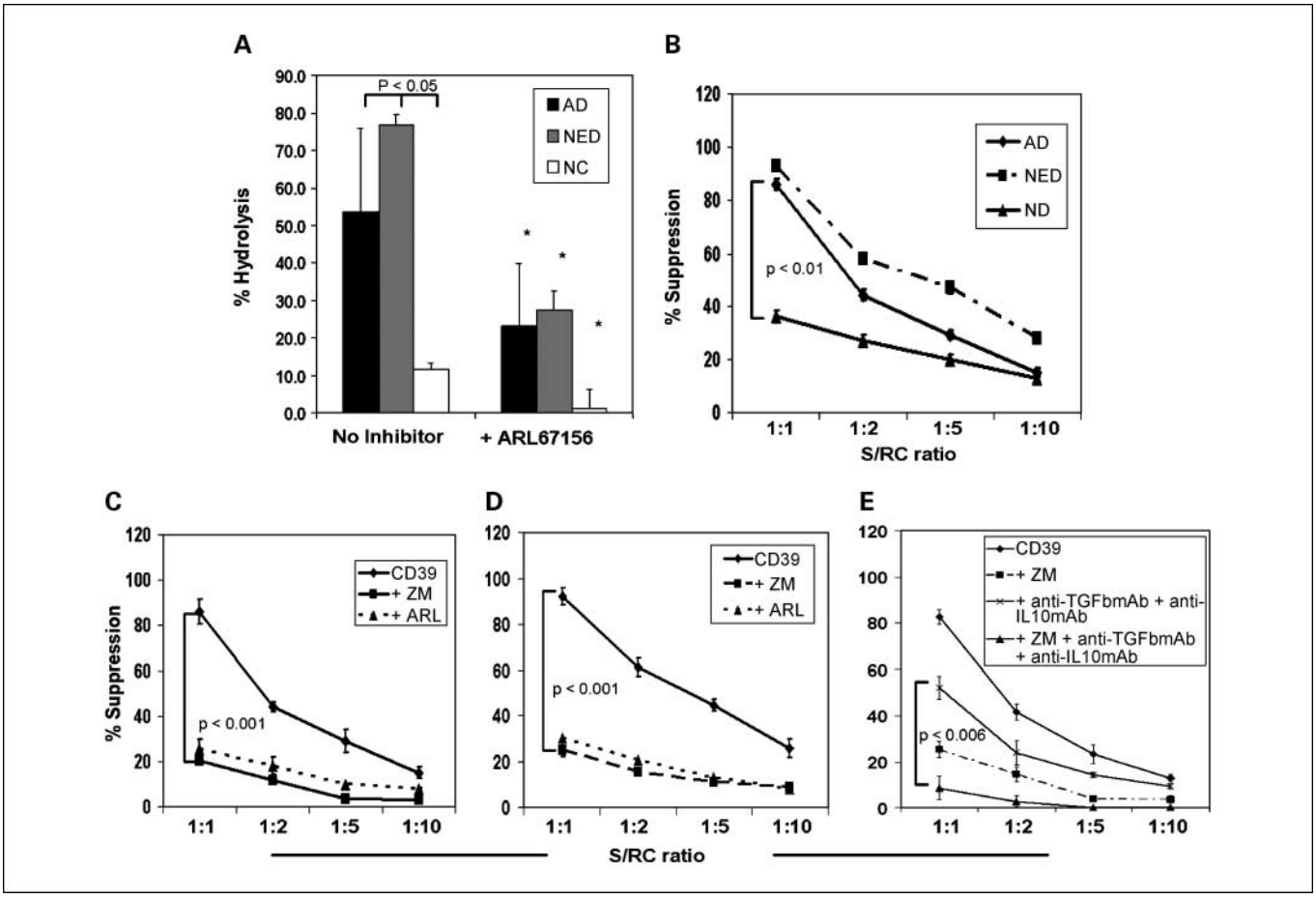

Fig. 4. Enzymatic activity of the ectonucleotidase CD39 in Treg obtained from HNSCC patients and normal controls and suppression mediated by $CD4^+CD39^+$ Treg with or without various inhibitors. *A,* single-cell–sorted $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cells were plated in 96-well plates (25,000 per well) in serum-free medium with 10 μmol/L exogenous ATP and unhydrolyzed ATP was measured after 30 min of incubation. The percent hydrolysis was calculated based on a standard curve. ARL67156 (selective ecto-ATPase inhibitor) was added to selected wells. *, $P < 0.01$, significant difference between cultures without and with the inhibitor. Mean ± SD of three independent experiments. *B,* MACS-sorted $CD4^+CD25^-$ cells (responder cells) were CFSE-labeled and stimulated with plate-bound OKT-3 and soluble anti-CD28 in the presence of $CD4^+CD39^+$ cells and 150 IU/mL IL-2 for 5 days. Cells were analyzed by flow cytometry and further analyzed using the ModFit program, gating on $CD4^+CFSE^+$ T-cell subsets. Suppression of $CD4^+CD25^-$ cell proliferation mediated by $CD4^+CD39^+$ cells at various responder/suppressor cell ratios isolated from normal controls and patients with cancer is shown. *C,* suppression of proliferation of responder cells in the presence of $CD4^+CD39^+$ cells isolated from patients with an active disease. To selected wells ZM241385, a selective A_{2a} and A_{2b} receptor antagonist, or ARL67156, an ectonucleotidase inhibitor, is added. *D,* suppression of proliferation of responder cells in the presence of $CD4^+CD39^+$ cells at various responder/suppressor cell ratios isolated from patients with no evident disease. To selected wells ZM241385 or ARL67156 is added. *E,* suppression of proliferating responder cells in the presence of $CD4^+CD39^+$ Treg pretreated or not with ZM241385, neutralizing anti-TGF-β1 and anti-IL-10 mAbs, or ZM241385 plus the two neutralizing mAbs. The *P* value is for differences in suppression levels between Treg treated with mAbs alone and Treg treated with mAbs plus ZM241385. Mean ± SD percent suppression (*B-E*) from three independent experiments.

Table 2. Adenosine production by Treg obtained from the peripheral blood of normal donors and HNSCC patients

	Adenosine measured (pg/μL)	
	Normal controls	Active disease
No inhibitor	131 ± 40	754 ± 147
+ ARL67156	35 ± 12	102 ± 21
+ α,β-Methylene ADP	10 ± 6	16 ± 8

NOTE: Cells were incubated with exogenous ATP (10 μmol/L) and supernatants were collected after 60 min. Adenosine concentrations were measured using mass spectrometry. ARL67156, an ectonucleotidase inhibitor (250 μmol/L/mL), or α,β-methylene ADP, a CD73 inhibitor (100 μmol/L/mL), was added to selected wells. Data represent three individual experiments.

$CD39^+$ Treg is in part mediated by the engagement of the $A_{2a}R$ on these effector cells and on the ability of Treg to produce immunosuppressive cytokines TGF-β1 and IL-10.

Discussion

In patients with cancer, Treg frequencies appear to correlate with the disease status and survival and seem to influence tumor progression (5, 6, 8, 24). Mechanisms known to be involved in enhanced Treg-mediated suppression seen in cancer patients include the involvement of immunosuppressive cytokines IL-10 and TGF-β, the production and secretion of perforin and granzyme B, and the expression of Fas/FasL on Treg (8, 25). Another potential mechanism of suppression involves ectonucleotidase-mediated production of adenosine, which has long been known as one of the most suppressive factors for immune cells (16–20, 26, 27). Although overexpression of ectonucleotidases in naturally occurring Treg has been recently described in normal controls and patients with multiple sclerosis (15), ours is the first study describing the involvement of ectonucleotidases in Treg-mediated suppression in cancer patients.

Several recent studies have emphasized the importance of the adenosinergic pathway in the process of tumor escape (19, 23, 28). Although ATP is known to inhibit tumor cell growth, as shown for pancreatic, colorectal, prostate, and esophageal cancers (29–32), ATP hydrolysis by ectonucleotidases CD39 and CD73 yielding adenosine facilitates tumor cell proliferation and invasion and inhibits antitumor functions of cytolytic lymphocytes, macrophages, and natural killer cells as well as accumulations of neutrophils (33–36). Adenosine also promotes neoangiogenesis by stimulating the proliferation of endothelial cells (37, 38) and vascular endothelial growth factor expression (39, 40). Further, the synthesis of proinflammatory cytokines IL-2 and tumor necrosis factor-α and adhesion of immune cells to the endothelium are inhibited by adenosine (41–43). In CD39-deficient mice, a significant inhibition of tumor angiogenesis and of endothelial and inflammatory cell migration into tumor tissues and pulmonary metastases has been observed relative to wild-type mice (22). These *in vivo* data emphasize the importance of extracellular nucleotidases in modulating various stages of the tumor pathogenesis.

Various tumor types have been shown to express ectonucleotidases on their cell surface and produce adenosine (44–46). It has been reported that appreciable amounts of adenosine are present in the interstitial fluid/tumor microenvironment of solid tumors at concentrations sufficient to inhibit cell-mediated immune responses to tumor cells (47). Interestingly, both tumor cells and Treg appear to employ the adenosinergic pathway for downregulating immune responses. The expression of CD39 and CD73 is upregulated on Treg present in the peripheral blood and tumor tissues of patients with HNSCC, which is in concert with an increased activity of these enzymes in Treg. Furthermore, $CD4^+CD39^+$ Treg accumulate in the tumor microenvironment, which is characterized by elevated levels of ATP due to the rapid proliferation and death of tumor cells. In this microenvironment, $CD4^+CD39^+CD73^+$ Treg are able to generate high levels of immunosuppressive adenosine. Also, extracellular adenosine enhances the generation of Treg as shown by us[8] and others (48). Adenosine mediates immunosuppression by binding to $A_{2a}R$ expressed on the surface of effector T cells (19, 20). $A_{2a}R$-deficient mice show enhanced antitumor immune responses mediated by T cells and a significant reduction in tumor growth compared with wild-type animals (23). In our study, blocking of these receptors on the surface of effector T cells decreased suppressor functions of Treg, confirming that the suppressive effects of Treg are mediated via $A_{2a}R$ signaling and subsequently by an increase in the level of intracytoplasmic cyclic AMP. However, it remains unclear whether $A_{2a}R$-mediated immune suppression has clinical significance or is implicated in cancer pathogenesis.

Experiments in murine models and our data strongly suggest that cancer progression of human cancer involves the up-regulation of the Treg-associated adenosinergic pathway. This study shows for the first time a significant correlation between CD39 expression on Treg and the disease stage in HNSCC. Increased CD39 expression (percent positive cells and MFI) in T cells of patients with cancer is likely related to accumulation of Treg in the tumor and peripheral circulation as reported previously by us and others (6–8). This Treg accumulation might be related to inflammatory infiltrates and proinflammatory cytokines present in the tumor microenvironment, including that of HNSCC (49). The characteristics of the tumor microenvironment, specifically cyclooxygenase-2 and prostaglandin E_2 expression, contribute to up-regulation of CD39 in Treg as indicated by our preliminary results.[9] Although observed in a relatively small patient cohort, a differential ectonucleotidase expression on Treg of HNSCC patients with various disease stages may prove to be a significant biomarker of disease prognosis in the future.

A significantly increased ectonucleotidase expression and activity was observed in Treg of HNSCC patients who received adjuvant radiochemotherapy compared with those treated with surgery alone, confirming previously published data by us in a cohort of HNSCC patients with no evident disease treated with radiochemotherapy (8). Both radiation and chemotherapy lead to chronic tissue inflammation and oxidative stress. Infiltration of Treg and their accumulation in inflamed/damaged tissues likely represent a normal physiologic response. Therefore, it is not surprising that radiochemotherapy can induce accumulations of $CD39^+$ Treg and enhance/maintain their enzymatic functions. During inflammation, ATP released from necrotic tumor cells as well as

[8] Unpublished data.
[9] M. Mandapathil, unpublished data.

various inflammatory cell types, including mast cells, lymphocytes, and macrophages, is rapidly degraded to adenosine. Increased CD39 expression on inflammatory cells in chronic inflammation and neoplastic formations has been reported previously (50) and might be a compensatory response to inflammation, pro-oxidative states, or radiochemotherapy of Treg in cancer patients. Future studies are needed to determine whether CD39 and CD73 expression is regulated by the tissue microenvironment (e.g., hypoxia) and whether it synergizes with cyclic AMP–induced signaling.

In summary, this study evaluates for the first time the involvement of adenosine in Treg-mediated immunosuppression in patients with HNSCC, which can be blocked by using $A_{2a}R$ antagonists and CD39 inhibitors. This knowledge could be important for the development of novel strategies aimed at enhancing the effectiveness of immune-based therapies for solid tumors. Potentially, application of $A_{2a}R$ antagonists in immune-competent cells and preventing CD39 activity on Treg and tumor cells, thus decreasing adenosine levels, might prove to be effective in enhancing antitumor immune responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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