Regulatory T Cells from Colon Cancer Patients Inhibit Effector T-cell Migration through an Adenosine-Dependent Mechanism

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Abstract

T cell–mediated immunity is a major component of antitumor immunity. In order to be efficient, effector T cells must leave the circulation and enter into the tumor tissue. Regulatory T cells (Treg) from gastric cancer patients, but not from healthy volunteers, potently inhibit migration of conventional T cells through activated endothelium. In this study, we compared T cells from colon cancer patients and healthy donors to determine the mechanisms used by Tregs from cancer patients to inhibit conventional T-cell migration. Our results showed that circulating Tregs from cancer patients expressed high levels of CD39, an ectoenzyme mediating hydrolysis of ATP to AMP, as a rate-lowering mechanism by which Tregs from cancer patients to inhibit conventional T-cell migration. Our results showed that circulating Tregs from cancer patients expressed high levels of CD39, an ectoenzyme mediating hydrolysis of ATP to AMP, as a rate-determining first step in the generation of immunosuppressive adenosine. Tumor-associated Tregs expressed even more CD39, and we therefore examined the importance of adenosine in Treg-mediated inhibition of T-cell transendothelial migration in vitro. Exogenous adenosine significantly reduced migration of conventional T cells from healthy volunteers, and blocking either adenosine receptors or CD39 enzymatic activity during transmigration restored the ability of conventional T cells from cancer patients to migrate. Adenosine did not directly affect T cells or endothelial cells, but reduced the ability of monocytes to activate the endothelium. Taken together, our results indicate that Treg-derived adenosine acts on monocytes and contributes to reduced transendothelial migration of effector T cells into tumors. This effect of Tregs is specific for cancer patients, and our results indicate that Tregs may affect not only T-cell effector functions but also their migration into tumors. Cancer Immunol Res; 4(3): 183–93. ©2016 AACR.

Introduction

Cytotoxic T cells and natural killer cells, supported by cytokine-secreting effector T-helper cells (Th), are the most important components of antitumor immunity (1). Tumor-associated antigens usually give rise to circulating CD8⁺ cytotoxic T cells in the affected patients, but in order to be efficient, effector T cells must leave the circulation and enter into the tumor tissue, as has been demonstrated in colon cancer where the infiltration of CD8⁺ and Th1-like CD4⁺ cells is clearly correlated to improved survival (2, 3). In contrast to CD4⁺ and CD8⁺ effector cell infiltration, accumulation of regulatory T cells (Treg) in different types of solid tumors induces immune-escape mechanisms (4, 5). In vitro, Tregs suppress the activity of conventional T cells in both a contact-dependent and -independent manner, and several suppressive mechanisms have been suggested, including surface expression of TGFβ and CTLA-4, transfer of cyclic adenosine monophosphate (cAMP), and adenosine hydrolysis from adenosine triphosphate (ATP; refs. 6–10). Several studies have demonstrated increased frequencies of circulating Tregs in patients with solid tumors, as well as a preferential accumulation of Tregs in tumors compared with surrounding tissue. Treg infiltration into tumors usually correlates with a poor prognosis (4), although their prognostic significance in colon cancer remains debated (11–13). Nevertheless, removal of Tregs during in vitro stimulation reveals tumor-specific T cell clusters in colon cancer (14–16). Together, these results implicate Tregs as important players in the inhibition of tumor-specific conventional T cells in colon cancer, and a better understanding of Treg function in intestinal tumors is essential to improve immunotherapies aimed at enforcing the patient’s own antitumor responses.

One way by which Tregs may contribute to reduced antitumor immunity is through reduced migration of conventional T cells into tumors. We have previously shown that Tregs from gastric and colon adenocarcinoma patients can inhibit in vitro migration of conventional T cells through endothelium activated by the bacterium Helicobacter pylori. In contrast, Tregs from healthy volunteers did not affect transendothelial migration (TEM; ref. 17). The effect was contact dependent, and did not rely on TGFβ or IL10 secretion.

During the past decade, it has become evident that Tregs can mediate adenosine hydrolysis from ATP through expression of
the ectonucleotidases CD39 and CD73 (9, 10, 18). The first, and rate-limiting, step in this process is mediated by CD39, which hydrolyses ATP to adenosine diphosphate (ADP) and AMP. Extracellular AMP will then rapidly be degraded to adenosine by CD73 (19). Generation of adenosine has emerged as an important mechanism for Treg suppression of proliferation and cytokine secretion (9, 10, 20, 21), and transplantation experiments have also confirmed the importance of Treg CD39 expression in vivo (10). Whereas mouse Tregs express both CD39 and CD73, human Tregs can be divided into CD39+ and CD39− subsets and have a lower expression of surface CD73 (10, 20, 21). Generation of adenosine is initiated in inflammatory conditions by both hypoxia-driven signaling pathways and the activity of Tregs. Hypoxia-inducible transcription factor 1α and tissue-derived extracellular adenosine act in concert to promote high expression of CD39 and CD73 on Tregs (19). Adenosine binding to inhibitory A2 adenosine receptors on T cells results in increased cAMP, which in turn suppressed the activity of effector T cells (22–24). The presence of adenosine also promotes the generation and expansion of Tregs during T-cell priming, and will decrease the Th1-promoting activity of dendritic cells (25, 26).

With regard to leukocyte migration, adenosine also regulates expression of adhesion molecules on cultured endothelial cells and enhances endothelial barrier function (27–29). CD39-dependent generation of adenosine suppresses T-cell and neutrophil accumulation in a murine model of contact hypersensitivity reactions, and adenosine also restricts lymphocyte migration through high endothelial venules in lymph nodes (27, 30). Based on these effects of adenosine on lymphocyte–endothelial cell interactions, we have investigated the expression of CD39 on tumor-associated Tregs and the effect of adenosine-mediated signals on T-cell migration, in order to better understand the mechanism by which Tregs from cancer patients inhibit T-cell migration.

Here we demonstrate that Tregs from colon cancer patients have the ability to reduce T-cell TEM and that Tregs from cancer patients express more CD39 than do Tregs from healthy individuals, especially tumor-associated Tregs. Treg-derived adenosine reduced TEM of T cells from healthy individuals, possibly acting through monocytes, and when adenosine receptors or CD39 enzymatic activity was blocked, the ability of conventional T cells from cancer patients to migrate through activated endothelium was restored.

Materials and Methods

Volunteers and isolation of lymphocytes

The study was performed with the permission of the Regional Research Ethics Committee of West Sweden, and informed consent was obtained from all participants. Peripheral blood was collected from 45 colon adenocarcinoma patients, 1 patient with an asymptomatic Swedish volunteer and was cultured for at least 3 years prior to colectomy, and none of the patients had undergone radiotherapy or chemotherapy for at least 3 years prior to colectomy, and none suffered from autoimmune diseases. Blood samples were collected in heparinized tubes, and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Paque (Pharmacia) density-gradient centrifugation. From 7 of the colon adenocarcinoma patients, a piece of the tumor and unaffected colon mucosa was collected at the time of surgery. The tissue was immediately placed in ice-cold PBS and used for isolation of lamina propria lymphocytes (LPL) by collagenase/DNase enzymatic digestion after removal of epithelial cells, as described (31).

H. pylori culture conditions

H. pylori strain Hel312 (cagPAI+ and vacA1tm1) was originally isolated from an asymptomatic Swedish volunteer and was cultured on Columbia-Isso agar plates as described (32). After 3 days of culture, bacteria were harvested from the plates by scraping and resuspended in PBS for optical density measurements at 600 nm. The final working concentration was adjusted with PBS to an optical density of 1.0, corresponding to approximately 5 × 10⁸ colony-forming units (CFU), prior to stimulation.

Transendothelial migration assay

TEM was analyzed using an in vitro Transwell chamber system essentially as described (17, 32). In brief, human umbilical vein endothelial cells (HUVEC; Cascade Biologies Inc.) were used at passage 4 to 6 for migration experiments and grown to confluence on fibronectin-coated filters in 6-well Transwell plates (Costar). CFU (5 × 10³) of H. pylori Hel 312, 100 ng/mL of TNFα, IL1β, and IL8 (all from Peprotech Ltd.), 1.0 and 0.1 μg/mL of lipopolysaccharide (LPS, Escherichia coli, serotype O26:B6), or 30 μg/mL of polyinosinic–polycytidylic acid potassium salt (polyo:C; both from Sigma-Aldrich) were added basolaterally 4 hours prior to the addition of 10⁸ PBMC at the apical side of the endothelial layer. Transmigration was then performed at 37°C, and after 16 hours migrating cells were collected from the lower chamber and characterized and enumerated by flow cytometry using True-Count beads (BD Biosciences). Migration was expressed as the percentage of CD4⁺ or CD8⁺ T cells from the starting PBMC suspension that was recovered in the lower well at the end of the experiment. In some experiments, the starting population was labeled with 1 μmol/L of 5-(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE; Invitrogen) for 30 minutes at 37°C and washed twice before the migration assays.

To assess the importance of adenosine-mediated signals during transmigration, 10 μmol/L of the stable adenosine analogue 2-chloro-adenosine (Tocris) was added to the migration assay at the start of the experiment, and was present all through the migration. Addition of 2-chloro-adenosine did not affect the viability of PBMCs or HUVECs. In other experiments, 25 μmol/L of the general adenosine receptor antagonist 1,3-dipropyl-8-(p-sulphophenyl)xanthine, 100 nmol/L of the A₂a adenosine receptor (A₂aR) antagonist SCH58261, 1 μmol/L of the A₂aR agonist CGS21680 (all from Sigma-Aldrich), or 10 μg/mL of a neutralizing antibody to the CD39 ectoenzyme (ref. 33; clone BY40/OREG103; Orega Biotech, Lyon, France) was added to the cells 30 minutes before the start of the migration.

In vitro stimulation and coculture experiments

CD4⁺ T cells were isolated using Dynabeads and Detachabeads from Dynal AS. They were subsequently stimulated with bead-bound antibodies to CD3 and CD28 (Invitrogen), or 1 or 0.1 μg/mL of erythropoemagglutinin (PHA-E) at 4 × 10⁶ cells/mL in round-bottomed 96-well plates for 24 hours in the presence or absence of 10 μmol/L 2-chloro-adenosine. HUVECs were seeded at 5 × 10⁵ cells/well in 24-well culture plates and cultured for 48 hours. They were stimulated with 1 μg/mL of E. coli LPS in the presence or absence of 10 μmol/L 2-chloro-adenosine for 6 and 20 hours. At these time points, culture medium was collected and cells were harvested by trypsinization. Cells were stained for flow...
cytometry, and chemokine concentrations were determined in the culture medium.

In the coculture experiment, HUVECs were seeded as above, and then cultured for 20 hours together with PBMCs isolated from healthy donors. In some experiments, CD4^+^, CD8^+^, or CD14^+^ cells were removed from PBMCs by Dynabeads (Dynal Biotech AS) before coculture. In other experiments, CD14^+^ monocytes were isolated by MACS magnetic microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. In some of the latter experiments, the isolated CD14^+^ monocytes were added to HUVEC cells either directly or following 1 hour of incubation with 10 μmol/L 2-chloro-adenosine in plates with ultra-low attachment surfaces (Corning). Monocytes incubated without 2-chloro-adenosine were used as control. Before harvest of endothelial cells, PBMCs were collected and HUVECs were released by trypsinization, followed by antibody staining for flow cytometry. HUVECs were distinguished from remaining PBMC by their CD31 expression.

Chemokine detection
CCL17 (TARC) and CCL22 (MDC) concentrations were determined by Duoset ELISA (R&D Systems). CCL5 (RANTES), CXCL8 (IL8), and CXCL10 (IP-10) were detected using cytometric bead array chemokine analysis (BD Biosciences).

Antibodies and flow cytometry analyses
Flow cytometry analyses were performed essentially as described (17), using the following antibodies and reagents: anti-CD4 (clone SK3), anti-CD8 (RPA-T8), anti-CD25 (2A3), anti-CD31 (WM59), anti-integrin α4 (CII.2R), anti-CD54 (HAS8), anti-CD62E (68-5H11), and anti-CD73 (AD2) from BD Biosciences, anti-FOXP3 (PCH101) from eBiosciences, anti-CD62E (68-5H11), and anti-CD73 (AD2) from BD Biosciences, anti-CD39 (A1) from AbD Serotec, CSFE, and 7-amino-actinomycin D (7AAD) from Sigma-Aldrich. Data acquisition was performed on a LSRII flow cytometer (BD Biosciences) equipped with FACSDiva software (BD Biosciences) and analyzed using FlowJo software (TreeStar Inc).

Flow cytometry cell sorting
Flow cytometry cell sorting was used for the deletion of CD4^+^CD25^high^ Tregs from the starting PBMC population in some TEM experiments. Cells were sorted using anti-CD4, anti-CD25, and anti-CD8 as described (17) on a FACSAria (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Tregs were identified by their high expression of CD25, and this population of CD4^+^CD25^high^ cells has previously been demonstrated to contain the Foxp3^+^ Tregs, both in healthy volunteers and cancer patients (31).

Statistical analysis
Statistical analyses were performed using a paired two-tailed Student t test when comparing data obtained from the same individual, and an unpaired t test if data were compared between cancer patients and controls. Values of P<0.05 were considered to be statistically significant.

Results
T cells from cancer patients have reduced TEM
We had previously established an in vitro system to study the TEM of T cells through HUVEC monolayers (32). Transmigrating cells are collected from the lower chamber, and characterized and enumerated by flow cytometry (Supplementary Fig. S1A). Our previous study demonstrated that live H. pylori bacteria induce a dose-dependent increase in T-cell TEM compared with unstimulated controls (32). To compare different migration-inducing stimuli of the endothelial cells, we now used ligands for Toll-like receptor (TLR) 3 and 4, expressed by HUVECs, as well as the inflammatory cytokines TNFα and IL1β and the chemokine IL8. All of the agents used induced a similar migration of both CD4^+^ and CD8^+^ T cells, with the exception of IL8, that only induced a very modest response (Supplementary Fig. S1B and S1C). Together, these results demonstrate that different endothelial stimuli can induce a similar T-cell migration response of both CD4^+^ and CD8^+^ T cells.

Because LPS and polyIC could induce TEM to the same extent as H. pylori, we continued to use these migration stimuli to expand our previous finding that T cells from cancer patients have reduced TEM capacity through endothelium activated by live bacteria compared with healthy volunteers (17). Consistent with previous results, live H. pylori induced only a minor migration of CD4^+^ T cells from cancer patients compared with CD4^+^ T cells from healthy volunteers, and there was no significant difference in LPS- and polyIC-induced migration compared with H. pylori-induced migration (Fig. 1A). CD8^+^ T cells from cancer patients also had a reduced migration capacity with all stimuli examined compared with their counterparts from healthy volunteers (Fig. 1B), although not as pronounced as for the CD4^+^ T cells.

To verify that the percentage of migrating cells was not influenced by subsequent cell divisions after migration, PBMCs were stained with CFSE prior to transmigration. Migrating cells did not divide, regardless of stimuli used, and virtually all migrating lymphocytes remained alive (7AAD) during the time of the experiment. These results show that T-cell TEM in cancer patients was reduced compared with healthy volunteers, regardless of the stimulus used to activate the endothelial cells.

Tregs suppress conventional T-cell TEM in colon cancer patients
To examine whether Tregs could influence LPS- and polyIC-induced TEM of T cells from colon cancer patients, CD4^+^CD25^high^ Tregs were depleted from the starting PBMC populations using flow cytometry cell sorting (Fig. 2A). In healthy volunteers, the migration of CD4^+^ T cells through LPS and polyIC-activated endothelium was neither changed by the removal of CD4^+^CD25^high^ Tregs nor when Tregs were put back to the suspension again prior to migration as a control (Fig. 2B). In contrast, when we removed Tregs from PBMCs collected from colon cancer patients, a significant increase in LPS and polyIC-induced CD4^+^ T-cell TEM was detected compared with when using the starting PBMC fraction (P<0.05 and P<0.01, respectively). In addition, when Tregs were added back to the Treg-free fraction, a significantly decreased CD4^+^ T-cell migration was detected (P<0.05 and P<0.01, respectively; Fig. 2C). Similarly, the depletion of Tregs from healthy volunteers did not influence CD8^+^ T-cell TEM in healthy volunteers (Fig. 2D), whereas LPS-induced CD8^+^ T-cell TEM in colon cancer patients was significantly increased after Treg removal (P<0.05; Fig. 2E). When Tregs were added back, the LPS-induced migration of CD8^+^ cells was reduced again.

The experiments described above were performed at the time of surgery, and thus in patients with detectable tumors. One year
after surgery, the same patients, with no detectable recurrence of disease, donated blood again. Transmigration experiments at this time demonstrated that T cells isolated from the patients 1 year after colectomy now had a restored ability to migrate through activated endothelium that was not significantly different from that of age-matched healthy controls (Fig. 3A and B). Taken together, these results show a significant increase in the migration of CD4$^+$ and CD8$^+$ T cells after Treg depletion in colon cancer patients, whereas no difference in TEM was observed when depleting Tregs from healthy volunteers. Furthermore, the migration potential of T cells from patients that had recovered from their colon cancer was similar to that of healthy volunteers.

**Tregs from cancer patients have increased expression of CD39**

We then decided to elucidate the mechanisms by which Tregs from cancer patients suppress TEM migration of T cells. It has been reported that adenosine affects TEM (27, 29, 30), and that CD39, an ectoenzyme involved in the generation of adenosine, is over-expressed by Tregs from human tumors (21, 34). We thus speculated that the CD39–CD73–adenosine pathway may mediate Treg-induced inhibition of TEM, and to test this hypothesis we analyzed the expression of CD39 and CD73 on CD4$^+$CD25$^-$, CD25$^{int}$, and CD25$^{high}$ circulating T cells by flow cytometry. These analyses demonstrated that significantly more CD4$^+$CD25$^{high}$ Tregs expressed CD39 in cancer patients than in healthy individuals ($P < 0.05$; Fig. 4A and B). Approximately 60% of the Tregs from cancer patients were CD39$^+$, whereas only about 30% of Treg from healthy individuals expressed CD39. The expression of CD39 on CD25$^{int}$ and CD25$^-$/CD4$^+$ T cells was substantially lower in both groups (Fig. 4A and B). Surface expression of CD73 was low in all the analyzed cell populations, as reported (21), and did not differ between patients and controls (Supplementary Fig. S2A). Consequently, very few cells coexpressed CD39 with CD73 (Fig. 4A). CD39 expression was also examined 1 year after surgery on cells from previous cancer patients. At this time, the CD39 expression on CD25$^{high}$ cells was reduced, and similar to that on Tregs from age-matched healthy controls (Fig. 4C).

To investigate CD39 expression in the local tumor microenvironment, we also isolated lymphocytes from tumors and unaffected colon tissue from the same patients and determined the expression of CD39 on Tregs and conventional T cells. These experiments showed that in the cancer patients, CD39 expression was similar on circulating Tregs and Tregs isolated from unaffected colon tissue. In contrast, virtually all tumor-infiltrating Tregs...
expressed CD39, and CD39 expression was significantly higher on tumor-infiltrating CD25high Tregs than on Tregs from unaffected colon (P < 0.001; Fig. 4D). As in blood, CD73 expression was low on colon and tumor-infiltrating Tregs (Supplementary Fig. S2B).

To further characterize CD39+ Tregs, we analyzed their expression of molecules important for migration and function of Tregs. The circulating CD39+ Tregs from cancer patients expressed CD62L, CCR8, and CD27 to the same extent as CD39− Tregs, but had a higher expression of CCR4 (92% ± 8% of CD39+ Tregs versus 63% ± 20% of CD39− Tregs, P < 0.01) and CCR6 (75% ± 9% versus 49 ± 13%, P < 0.01). In addition, the expression of CTLA-4 was substantially higher in CD39+ Tregs from both cancer patients and healthy controls compared with CD39− Tregs from the two groups (P < 0.01; Fig. 4E).

Because Tregs from cancer patients more or less lack CD73 expression, we tried to identify what other cell types may contribute to the last step in adenosine hydrolysis by analyzing CD73 expression on PBMC and HUVEC. Indeed, both monocytes and HUVECs uniformly express CD73 (Supplementary Fig. S3) and would therefore be able to convert AMP to adenosine.

Taken together, these data demonstrate that frequencies of CD39+ Tregs expressing CCR4, CCR6, and CTLA-4 are increased systemically in cancer patients compared with healthy controls, and that CD39+ Tregs accumulate further in colon tumors. These Tregs presumably contribute to generation of adenosine in the tumor microenvironment, and adenosine may thus be the factor produced by Tregs, isolated from colon cancer patients, resulting in reduced TEM.

**Adenosine inhibits TEM of conventional T cells**

To directly examine the effects of adenosine on TEM, 10 μmol/L of 2-chloro-adenosine was added to transmigration assays. These
experiments demonstrated that adenosine strongly reduces transmigration of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from healthy individuals (P < 0.05; Fig. 5A). In contrast, adenosine only had a minor effect on the migration of monocytes, which are also present in the PBMC fraction used in the migration experiments (P. Sundström, unpublished observations). Additional experiments were then performed with a selective agonist to A<sub>2A</sub>R, CGS21680. The effect of the A<sub>2A</sub> agonist was similar to that of 2-chloro-adenosine, suggesting that the adenosine effect on TEM may be mediated through A<sub>2A</sub>R (Supplementary Fig. S4A). To investigate if blocking of adenosine-generated signaling could restore TEM in cancer patients, we blocked the enzymatic activity of CD39 using a neutralizing antibody (33) during the transmigration process. Blocking of adenosine receptors during transmigration resulted in substantially increased TEM by PBMCs from 6 out of 8 cancer patients compared with untreated migration assays, but due to the large variation the results did not reach statistical significance (Fig. 5B). The effect was similar in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These results were extended using the selective A<sub>2A</sub>R antagonist SCH58261, which increased T-cell migration in some, but not all, cancer patients (Supplementary Fig. S4B). The reason for these somewhat inconclusive results may be that there is not enough adenosine generated in some of the cultures, and thus blocking of adenosine signaling would not have any effect. From these experiments, it cannot be unambiguously concluded that A<sub>2A</sub>R alone mediate the effect of adenosine. Finally, to confirm that the enzymatic activity of CD39 contributed to adenosine generation in cancer patient Tregs, we blocked CD39 activity using a neutralizing antibody (33) during the transmigration process. Blocking of CD39 activity also increased migration of effector T cells from cancer patients (Fig. 5C), strongly indicating that CD39 activity contributes to adenosine-mediated reduction of TEM in cancer patients.

Effect of adenosine on endothelial cells, T cells, and monocytes

We have previously identified intercellular adhesion molecule-1 (ICAM-1) and CCL5 as important mediators of T-cell migration in our Transwell system (17, 32). To delineate the effect of adenosine on adhesion molecules and chemokines in the TEM assay containing lymphocytes, monocytes, and endothelial cells, the different cell types were treated individually with adenosine to assess its effects on functions relevant for TEM. First, isolated CD4<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 or with PHA-E, and the effect of adenosine on adhesion molecule and chemokine receptor expression assessed. However, adenosine had negligible effects on T-cell expression of the ICAM-1 receptor integrin α<sub>4</sub>β<sub>2</sub> as well as the vascular cell adhesion molecule-1 (VCAM-1) receptor integrin α<sub>4</sub>β<sub>1</sub>. Furthermore, expression of chemokine receptors CCR5 (CCL5 receptor), CCR6, CCR9, and CXCR3 was not changed by treatment with adenosine.

To assess the effect of adenosine on HUVECs, we analyzed the expression of adhesion molecules ICAM-1, VCAM-1, and E-selectin, as well as chemokine secretion following LPS stimulation with and without adenosine. Treatment with 1 μg/mL of LPS strongly increased ICAM-1 and E-selectin expression on HUVECs, but had little effect on VCAM-1. HUVECs express A<sub>2A</sub>R (35), and the presence of 2-chloro-adenosine during stimulation reduced the E-selectin expression by 52% ± 20% 20 hours after stimulation, but had no effect on ICAM-1 expression. Activation by LPS also induced secretion of large amounts of the chemokines CCL5 (5.1 ± 2.0 ng/mL) and CXCL10 (15 ± 4.2 ng/mL), some CXCL8 (64 ± 35 pg/mL) but no CCL17 or CCL22. The addition of 2-chloro-adenosine reduced CCL5 and CXCL8 levels somewhat, but the effect was not statistically significant.

As expression of ICAM-1 and CCL5, or their receptors integrin α<sub>4</sub>β<sub>2</sub> and CCR5, was not changed during adenosine treatment of isolated T cells or HUVECs, we performed coculture experiments with PBMCs and HUVECs, to mimic the conditions during transmigration. We found that coculture of PBMCs and HUVECs increased the expression of ICAM-1 on endothelial cells (Fig. 6A), but had no effect on adhesion molecule expression by T cells. Furthermore, the endothelial ICAM-1 expression induced by coculture was dependent on the presence of monocytes, but not if the monocytes had been pretreated with 2-chloro-adenosine before coculture with HUVECs. Monocyte coculture with the endothelial cells induced a high surface expression of ICAM-1 on endothelial cells, but if the monocytes had been pretreated with 2-chloro-adenosine, this effect was substantially reduced (P < 0.001; Fig. 6C).

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**Figure 3.** TEM is restored 1 year after successful surgery. PBMCs were isolated from healthy volunteers and former colon cancer patients 1 year after relapse-free tumor surgery. The number of migrating CD4<sup>+</sup> T cells (A) and CD8<sup>+</sup> T cells (B) from healthy volunteers (●) and former colon cancer patients (▲) was analyzed by flow cytometry. The percentage of migrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells is expressed as individual data of four to five experiments. ns, not statistically significant.
Taken together, these results are consistent with a model in which Treg-derived adenosine acts primarily on monocytes and reduces their ability to stimulate HUVECs properly. This will in turn result in lower expression of endothelial ICAM-1 and reduced T-cell migration.

**Discussion**

Local T-cell responses are crucial for antitumor immunity (2, 3), and we show here that Tregs from colon cancer patients potently inhibit TEM of CD4+ and CD8+ T cells. The reduced migration is independent of T-cell apoptosis or proliferation, but controlled by...
Adenosine signaling. Tregs from cancer patients expressed high levels of the ecto-enzyme CD39 that promotes hydrolysis of ATP to AMP, in the first step of the cascade generating adenosine, and our in vitro studies suggest that adenosine acts on monocytes and reduce TEM of conventional T cells.

The results in the present study confirm and extend our previous findings that Tregs from gastric cancer patients inhibit TEM of conventional T cells. We could first establish that this inhibition was seen after activation of endothelial cells with different stimuli, because neither live H. pylori bacteria nor TLR ligand stimulation could improve the poor migration of T cells from cancer patients. Instead, removal of Tregs from the lymphocyte suspensions from cancer patients restored the migration. Interestingly, depletion of Tregs from cell suspensions from the healthy controls did not affect the inhibition of T-cell migration. This indicates that it is not only the higher number of circulating Tregs in cancer patients that give rise to the inhibition of T-cell transmigration, but that there are also qualitative differences in the function of circulating Tregs in cancer patients versus healthy individuals.

During the past few years, it has become increasingly clear that the CD39-mediated first step in the hydrolysis cascade of ATP to adenosine is an important effector mechanism for human Tregs (9, 10, 20, 21). CD39<sup>+</sup> human Tregs seem to be particularly important for suppression of Th17 immune responses in autoimmunity, but have also been detected in patients with head and neck squamous cell carcinoma and in colon tumors (20, 21, 34). Furthermore, it was previously shown that Tregs suppress migration of T cells to the skin in a model of delayed-type hypersensitivity, and that this effect was mediated through adenosine (27). In this study, we show that circulating Tregs from colon cancer patients have a substantially higher expression of CD39 than that of healthy controls. We can also confirm recent findings by Scurr and colleagues (34) showing that virtually all Tregs present in colon tumors expressed CD39. Whether this is caused by local induction of CD39 in the tumor microenvironment or a selective recruitment of CD39<sup>+</sup> Tregs to the tumor is not clear. However, the increased expression of chemokine receptors CCR4 and CCR6 on tumor-associated CD39<sup>+</sup> Tregs may offer support for the latter possibility, especially because colon tumors show increased production of the CCR4 ligand CCL22 and the CCR6 ligand CCL20 (MIP-3α; refs. 36, 37). Based on the finding of increased CD39 expression in Tregs from cancer patients, we assessed the ability of the stable adenosine analogue 2-chloro-adenosine to inhibit TEM of T cells from healthy volunteers. The results of these experiments clearly demonstrated that adenosine reduces conventional T-cell TEM, and has effects on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Blocking of adenosine receptor signaling restored migration of T cells in most of the cancer patients up to the level of healthy volunteers, and selective blocking experiments indicate that A<sub>2A</sub>R may be involved in the signaling cascade, but also the possibility of contribution from other adenosine receptors. Blocking CD39 activity also increased migration of T cells from cancer patients, suggesting that CD39 activity is an important prerequisite for adenosine generation in our system. As Tregs from healthy individuals also express some CD39, there may be additional functions, apart from faster generation of adenosine, in the Tregs from cancer patients that make them particularly suited to suppress T-cell migration. For example, our observation of increased expression of CTLA-4 in CD39<sup>+</sup> Tregs suggests that they may also potently reduce antitumor immune effector functions in an adenosine-independent manner (7). To this end, tumor-associated CD39<sup>+</sup> Tregs also coexpress CTLA-4 (34). It has also been shown that effector T cells from cancer patients express less
adenosine deaminase (ADA) than corresponding cells from healthy volunteers, which would result in an accumulation of adenosine due to reduced deamination to inosine (38). In addition, effector T cells from cancer patients were more sensitive to inhibitory signals delivered via adenosine receptors. Thus, traits in both Tregs and effector T cells may act in concert to generate the substantial decrease in TEM documented in cancer patients.

Adenosine had no direct effect on T-cell or endothelial expression of adhesion molecules or chemokines, but instead potently reduced the ability of monocytes to induce endothelial ICAM-1 expression. As ICAM-1 is crucial for T-cell TEM (32), lack of monocyte signaling to the endothelium may be the key to reduced migration in the presence of adenosine. It is not clear from our results which monocyte-derived factors induce endothelial ICAM-1, but TNFα may be a candidate, as it is downregulated by adenosine treatment of monocytes (39).

The effect of cell-cell interactions may also be important, as monocyte interaction with endothelial cells promotes subsequent transmigration due to CD31 accumulation at endothelial junctions (40).

It is well established that Tregs accumulate in the blood and tumors of patients with colorectal cancer (14, 41), and Tregs from colon cancer patients can suppress specific immune responses to tumor antigens (14–16). Interestingly, in colon cancer the presence of Tregs in the tumor correlates with an improved prognosis (11, 12), in contrast to several other solid tumors, where the presence of Tregs is associated with more severe outcomes (4). One potential reason for these seemingly contradictory findings may be the high microbial load in direct contact with colon tumors (42). Microorganisms promote innate inflammation and Th17 activation, which in turn drive angiogenesis and tumor growth. Thus, if these responses are downregulated by Tregs, it may well be beneficial for the patient, as recently suggested by Ladoire and colleagues (43). However, ATP hydrolysis to adenosine in the

Figure 6.
Monocytes affect endothelial ICAM-1 expression and lymphocyte TEM. A, HUVECs were cultured alone (white bar) or with the whole PBMC fraction from healthy volunteers (black bar) for 16 hours, and the ICAM-1 expression on HUVECs was determined by flow cytometry and is given as mean fluorescence intensity (MFI). In other cultures, HUVECs were cocultured with PBMCs depleted of CD4⁺, CD8⁺, or CD14⁺ cells, respectively (gray bars) or with isolated CD14⁺ monocytes (dark gray bar). Scale bars, means ± SEM of seven to 13 experiments, and the value obtained with unfractionated PBMCs was set at 100%. B, migration of CD4⁺ (white bars) and CD8⁺ T cells (black bars) from healthy volunteers, using the whole PBMC fraction or PBMCs depleted of CD14⁺ cells, was analyzed by flow cytometry. The percentage of migrating CD4⁺ and CD8⁺ T cells is expressed as mean ± SEM of six experiments. C, HUVECs were cultured alone (white bar) or with isolated CD14⁺ monocytes that were untreated (dark gray bar) or treated with 2-chloro-adenosine (light gray bar). Left, individual values of ICAM-1 MFI; right, data expressed as mean ± SEM of five experiments, with the value obtained with untreated monocytes set at 100%.

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tumor microenvironment probably contributes to tumor progression by direct immunosuppressive effects on local T cells (22, 23, 44) and by stimulating angiogenesis (45, 46). Murine studies show that signaling through A2AR in T cells protects tumors from T-cell–mediated rejection, while deletion of CD39 on Tregs or treatment with CD39-neutralizing antibodies promotes antitumor cytotoxicity in experimental systems (24, 33, 47). In addition to these effects of infiltrating Tregs, our current results indicate that adenosine-mediated inhibition of TEM of effector T cells may be another important factor for tumor progression. We thus suggest a model in which Tregs mediate hydrolysis of ATP to AMP through CD39 activity, and even though human Tregs express little CD73, AMP may subsequently be degraded to adenosine by CD73 present on monocytes, endothelial cells, and tumor cells (48). Adenosine may then act on monocytes, which in turn will reduce ICAM-1 expression on the endothelium, leading to reduced T-cell TEM. Endothelial ICAM-1 expression correlates with T-cell infiltration in colon tumors (49), and the ability of Tregs to inhibit endothelial ICAM-1 upregulation in tumors has also been seen in vivo in studies examining T-cell homing to tumors (50). A recent study showed that reduced adenosine signaling during hyperoxic conditions improves infiltration of activated CD8+ T cells into tumors (51). Treg infiltration is also increased in this setting, but the net effect of hypoxia is still weaker immunosuppression by Tregs and improved tumor regression. Taken together, our results indicate that Treg activity in cancer patients may have potent effects not only on lymphocytes but also on myeloid and nonhematopoetic cells that would contribute to functional immune suppression locally in the tumors by reducing effector T-cell infiltration.

Disclosure of Potential Conflicts of Interest

J. Bastid has an ownership interest (including patents) in Orega Biotech. No potential conflicts of interest were disclosed by the other authors.

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References

Treg Mediated Inhibition of T-cell Migration in Cancer Patients


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