Suppressive effects of tumor cell-derived 5′-deoxy-5′-methylthioadenosine on human T cells

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ABSTRACT
The immunosuppressive tumor microenvironment represents one of the main obstacles for immunotherapy of cancer. The tumor milieu is among others shaped by tumor metabolites such as 5′-deoxy-5′-methylthioadenosine (MTA). Increased intratumoral MTA levels result from a lack of the MTA-catabolizing enzyme methylthioadenosine phosphorylase (MTAP) in tumor cells and are found in various tumor entities. Here, we demonstrate that MTA suppresses proliferation, activation, differentiation, and effector function of antigen-specific T cells without eliciting cell death. Conversely, if MTA is added to highly activated T cells, MTA exerts cytokotoxic effects on T cells. We identified the Akt pathway, a critical signal pathway for T cell activation, as a target of MTA, while, for example, p38 remained unaffected. Next, we provide evidence that MTA exerts its immunosuppressive effects by interfering with protein methylation in T cells. To confirm the relevance of the suppressive effects of exogenously added MTA on human T cells, we used an MTAP-deficient tumor cell-line that was stably transfected with the MTAP-coding sequence. We observed that T cells stimulated with MTAP-transfected tumor cells revealed a higher proliferative capacity compared to T cells stimulated with Mock-transfected cells. In conclusion, our findings reveal a novel immune evasion strategy of human tumor cells that could be of interest for therapeutic targeting.

Introduction
Tumors employ multiple mechanisms to evade immune surveillance, including downregulation of antigen presentation, expression of co-inhibitory molecules or secretion of immunosuppressive cytokines.1-4 Additionally, tumors can take advantage of their metabolic alterations to inhibit immune responses. The most prominent example is the so-called “Warburg Effect,” which describes the increased uptake and utilization of glucose by tumor cells,5 thereby facilitating rapid proliferation by relocation of glycolytic intermediates toward pathways used to synthesize lipids, nucleic acids and amino acids.6,7 We and others have demonstrated, that lactic acid, a prominent product of glycolysis generated in increased amounts by tumor cells, is capable of modulating cellular components within the tumor stroma, including immune effector cells and antigen-presenting cells.8-11

There is an increasingly recognized list of metabolic dysregulations linked to carcinogenesis. Various tumor entities, particularly malignant melanoma, leukemia, and hepatocellular carcinoma, can exhibit a reduced expression of 5′-deoxy-5′-methylthioadenosine phosphorylase (MTAP).12,13 MTAP deficiency in human tumors has been mainly attributed to promoter hypermethylation or to deletions and translocations of the chromosomal 9p21 region.12-15 The MTAP gene is localized in direct proximity to tumor suppressor genes such as cyclin-dependent kinase inhibitors p16INK4a and p15INK4b 16 and therefore has been the focus of several cancer-related studies.17 The MTAP enzyme plays a major role in polyamine
metabolism and in the methionine salvage pathway. It is constitutively expressed in almost all cells and tissues. MTA deficiency leads to increased invasiveness and poor response to adjuvant therapy with Interferon-α (IFN-α). MTAP catalyzes 5′-deoxy-5′-methylthioadenosine (MTA) degradation to adenine and 5′-methylthioribose-1-phosphate, which are subsequently converted to adenosine and methionine. Removal of accumulating MTA by MTAP is essential for the maintenance of methionine and polyamine regeneration. A decreased expression of MTAP leads to MTA accumulation within tumor cells and their microenvironment. The effect of MTA on tumor cells remains controversial: while intracellular concentrations of 0.5–5 μM MTA seem to promote tumor progression, higher concentrations of MTA interfere with cell proliferation, tumor development, invasiveness and regulation of apoptosis.

MTA has been shown to be toxic to hematopoietic progenitor cells in vitro with an IC_{50} equal or less than 1 μM. In contrast, MTA has been tested in mice and rats and found to be non-toxic at high doses even when given over extended periods. One further potent biochemical mechanism of MTA is its inhibition of methylation. Two recent publications postulate that the loss of MTAP in tumors leads to a heightened susceptibility to a depletion of the methyltransferase PRMT5, as increased intracellular MTA impedes PRMT5 activity.

Little is known about the effects of MTA on immune cells. Studies suggest an anti-inflammatory effect of MTA, because it inhibits both the secretion of pro-inflammatory cytokines and the activation of the pro-inflammatory transcription factor NF-κB, as was also shown for T cells. Inhibition of protein methylation appears to play a critical role in MTA’s inhibitory effect on pro-inflammatory mediators. Furthermore, MTA exerts inhibitory effects on lymphocyte function and proliferation and natural killer cell-mediated cytotoxicity, respectively.

Here, we address the question of whether MTA accumulation in MTAP-deficient tumors plays a role in the suppression of human antitumor immune responses. We demonstrate that MTA suppresses T cell activation and effector function. Its inhibitory effect is most likely mediated by reduced Akt phosphorylation and can be mimicked by other protein methylation inhibitors. A better understanding of MTA-mediated immunosuppression may help to improve the efficacy of emerging T cell-based cancer immunotherapies.

**Results**

**MTA inhibits the proliferation of human CD4+ and CD8+ T cells and the induction of cell cycle progression**

The fact that tumor cells secrete MTA into the microenvironment raised the question whether MTA exerts an effect on tumor-infiltrating lymphocytes. Indeed, MTA was found to suppress equally the proliferation of human CD4+ and CD8+ T cells after anti-CD3/CD28 stimulation in a dose-dependent manner (Fig. 1A). To address the question whether the inhibitory effect of MTA on anti-CD3/CD28 stimulated T cells is reversible, CFSE-labeled T cells were stimulated with anti-CD3/CD28 coated beads in the presence or absence of 100 μM MTA. After 7 d, medium was changed and T cells were restimulated with anti-CD3/CD28 in fresh complete RPMI medium without MTA for 5 more days. Fig. 1B demonstrates that T cells regain their capacity to proliferate after removal of MTA, indicating that inhibition of proliferation by MTA is reversible. This is consistent with the published data.

It remained unclear whether resting T cells are more sensitive to the immunomodulatory effects of MTA than stimulated T cells. To test this, freshly isolated T cells were either stimulated with anti-CD3/CD28 coated beads or not in the absence or presence of 100 μM MTA. After 7 d, we observed a clear decline in viability of stimulated versus non-stimulated T cells cultivated in the presence of MTA. Interestingly, the latter did not differ markedly from cells stimulated in the absence of MTA and only slightly from non-stimulated cells grown in the absence of MTA (Fig. 1C). We performed further kinetic studies to evaluate when and at which concentration the addition of MTA induces apoptosis (Fig. S1). MTA did not reduce the viability of T cells stimulated with anti-CD3/CD28 coated beads until day 3 (Fig. S1A). For unstimulated T cells, 50 μM and 100 μM MTA did not reduce the percentage of viable cells up to day 6 (Fig. S1B).

To test whether MTA blocks the cell cycle of T cells, freshly isolated T cells were stimulated with anti-CD3/CD28 and analyzed for the expression of cell cycle markers by flow cytometry. As demonstrated in Fig. 1D, both Ki-67 and Cyclin E, which promote transition from G1 to M phase, were significantly downregulated. Levels of the inhibitory cell cycle protein p16 (Fig. 1D), p27Kip1 (Fig. S2) and the anergy-associated molecule DGK-α (Fig. 1D) were decreased as well as the expression of the checkpoint molecules, CTLA-4, LAG-3 and Tim-3 (Fig. S2). Therefore, MTA inhibits the induction of proliferation in T cells and induces cell death in already activated T cells.

**Influence of MTA on effector functions of CTLs**

Next, we studied the influence of MTA on Mart-1-specific CTL functions. CTLs were co-cultured with Mart-1 (HLA-A2-restricted, ELAGIGILTV)-loaded T2 target cells at various E:T ratios in the presence or absence of MTA. We observed a 50% reduction of Mart-1-specific CTLs cytotoxicity in the presence of 500 μM MTA (Fig. 2A). Correspondingly, MTA-treated CTLs expressed less CD107a, a degranulation marker, in a dose-dependent manner (Fig. 2B and S3). In addition, we observed a dose-dependent inhibition of IFN-γ and IL-2 production by CTLs upon antigen recognition at 250 μM and 500 μM MTA (Fig. 2C), with a corresponding decrease in the frequency of IL-2 and IFN-γ-producing cells of 60% (for 250 μM) and 80% (for 500 μM), respectively (Fig. 2C and D).

**MTA prevents in vitro induction of antigen-specific CTLs**

Next, we were interested in the ability of MTA to inhibit the in vitro induction of human antigen-specific CTLs. To that end, human CD8+ T cells were magnetically enriched from PBMCs and stimulated with autologous Mart-1-loaded moDCs. CD8+ T cells were cultured in the presence or absence of 25 and
The frequency of Mart-1-specific CTLs on day 11, as determined by Mart-1-multimer staining, rose to 25.6% of total CTLs in the control group (without MTA) (Fig. 3A and B). In contrast, induction of antigen-specific T cell responses was impaired and even completely abrogated in the presence of 25 and 50 μM MTA, respectively, with frequencies of Mart-1 multimer+ CTLs ranging from 0.1–1.9% on day 11 (Fig. 3A and B). Subsequently, we asked whether MTA reduced the absolute number of antigen-specific CTLs and suppressed the expansion of antigen-non-specific CTLs. For this, CTLs were cultured for 18 d in the presence or absence of 10 to 50 μM MTA. We observed a dose-dependent inhibition of the expansion of Mart-1 multimer+ CTLs with a total inhibition at 50 μM MTA (Fig. S4). The expansion of CTLs, which were not Mart-1 multimer-positive but bystander-stimulated, was also impaired, albeit to a lesser non-significant degree.

Interestingly, despite their stimulation with antigen-pulsed moDCs in the presence of IL-2, T cells treated with MTA did not upregulate activation molecules such as CD25, CD38, CD54, CD69, CD71, and CD137 (only shown for CD25, CD69 and CD137) and kept their naive phenotype (low expression of...
CD45RO and high expression of CD27 and CCR7) (Fig. 3C). In contrast, T cells not treated with MTA showed significant upregulation of the activation molecules and downregulation of CD27 and CCR7. Interestingly, the checkpoint molecule PD-1 was not up-regulated (Fig. S5).

Next, we determined whether MTA is capable of suppressing highly activated antigen-specific CTLs. Thus, CD8\(^+\) T cells were stimulated weekly with Mart-1-pulsed autologous moDCs. After two cycles of stimulation, CTLs were cultured in the presence or absence of 25 and 50 \(\mu\)M MTA, respectively, for 1 week. The percentage of Annexin-V/7-AAD double positive Mart-1-specific T cells had increased to 30.3% compared to 9.1% in the control group (Fig. 3D). In parallel, the percentage of Mart-1-specific CTLs had decreased from 30.3% to 13.5% in the presence of 50 \(\mu\)M MTA, while in the absence of MTA the percentage had increased from 30.3% to 48.4% (Fig. 3D). Taken together, already small concentrations of MTA increase the rate of apoptosis in an antigen-specific CD8\(^+\) T cell co-culture system. Moreover, T cells activated by antigen-specific stimulation are more susceptible to the effects of MTA compared to non-specific T cells activated by bystander effects (e.g. IL-2).

**Influence of MTA on effector functions of CD4\(^+\) T cells**

MoDC-stimulated CD4\(^+\) T cells cultured in the presence or absence of 25 and 50 \(\mu\)M MTA, respectively, revealed no differences in the percentage of CD25\(^{high}\), FoxP3\(^+\), CD127\(^-\) CD4\(^+\) T\(_{reg}\) cells (Fig. 4A). However, several cytokine responses of CMV-reactive CD4\(^+\) T cells were diminished after antigen-specific stimulation in the presence of MTA, albeit reaching significance only at the concentration of 50 \(\mu\)M MTA (Fig. 4B). The secretion of both T\(_{H1}\) cytokines (IFN-\(\gamma\), Lymphotoxin-\(\alpha\)) and T\(_{H2}\) cytokines (IL-4, IL-5) was suppressed. Only IL-8 was not downregulated. As this cytokine is mostly secreted by DCs,
this is an indication that MTA does not inhibit mature moDCs in their capacity to activate T cells. Taken together, these data clearly indicate that concentrations of MTA as low as 25 μM are able to suppress human CD4+ and CD8+ T cell responses, but they do not induce T<sub>reg</sub> cells.

**MTA interferes in highly activated T cells with the Akt pathway**

TCR stimulation activates multiple distal pathways including the MAPK pathways Erk and p38, as well as NF-κB and NFAT pathways, which are additionally controlled through Akt. Thus, we analyzed the impact of MTA on early T-cell-signaling events. For this purpose, we used prestimulated antigen-specific CTLs as highly activated effector T cells. T cells were rested in medium with or without 50 μM MTA. Signaling events were then induced by PMA/ionomycin stimulation. Several pathways (growth, cell cycle, survival, stress and damage) were analyzed by the PathScan®-technology. Of interest, preincubation with MTA affected the Akt phosphorylation and the phosphorylation of molecules downstream of Akt (mTOR, S6 and GSK-3β) (Fig. 5A). Equally, Erk and Stat phosphorylation was impaired (Fig. 5A). The detection of a higher cleavage of the apoptosis-associated molecules PARP and Casp3 strengthened the finding, that MTA induced apoptosis in highly activated T cells.

The slower and significantly lower increase in phosphorylated Akt upon stimulation of MTA-treated cells compared to...
untreated controls was confirmed by flow cytometry (Fig. 5B). Of interest, phosphorylation of Akt was downregulated in MTA-treated T cells already after the 24-h preincubation period as compared to the controls (Fig. 5B). MTA did not reduce the overall expression of Akt and Erk1/2 (Fig. S6). The phosphorylation of p38 was not influenced by MTA (Fig. 5A).
and data not shown). We also observed that MTA did not influence the downstream Ca\(^{2+}\)-influx upon stimulation of T cells (data not shown).

**Mechanism of T cell inhibition**

Next, we asked whether MTA inhibition could be due to its adenosine residue, because adenosine is a common inhibitor of T cell function in the tumor microenvironment. We preincubated T cells with dipyridamole, a blocker of adenosine uptake. Dipyridamole was not able to counteract inhibition of T cells by MTA. In contrast, adenosine-mediated suppression of T cell activation was completely abrogated (Fig. 6A). Excluding that MTA is further degraded into adenosine, we treated CD8\(^{+}\) T cells with erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), a strong inhibitor of adenosine deaminase, in combination with adenosine and/or MTA and analyzed T cell activation by flow cytometry. Adenosine-mediated inhibition of T cell activation was enhanced by EHNA, whereas EHNA had no additive effect on T cell repression by MTA (Fig. S7A). Further, we demonstrated that MTA did not lead to an increase of intracellular cAMP-levels (Fig. S7B). Hence, MTA does not inhibit T cells by its adenosine residue. To determine MTA degradation by T cells, we analyzed MTA and related metabolites in the supernatant of moDC/CD8\(^{+}\) T cell co-cultures at various time points. Fig. 6B shows that MTA is relatively stable in medium without cells over a period of 8 d. In a coculture of moDCs and CD8\(^{+}\) T cells, in contrast, the concentration of MTA dropped by half with a contaminant increase in the concentration of the MTA metabolite adenine, for the first time indicating that T cells are capable of metabolizing MTA.

MTA is able to interfere with transmethylation reactions either directly or through inhibition of S-adenosyl-L-homocysteine hydrolase. The effects of 25 \(\mu\)M MTA on the induction of antigen-specific T cells and T cell apoptosis were analyzed by adding 3'-deazaadenosine (3-Deaza), an S-adenosyl-L-homocysteine hydrolase inhibitor, 5'-aza-2'-deoxycytidine (Aza-CdR), a DNA-methylation inhibitor, and
3′-deazaneplanocin A (DZNep), a specific EZH2-methylation inhibitor, to a co-culture of CD8+ T cells and peptide-pulsed moDCs. All inhibitors are nucleoside-derivatives and were used at equimolar concentrations for better comparison. Apoptosis and antigen specificity were measured on day 11. Aza-CdR and DZNep were toxic to T cells yielding high rates of T cell apoptosis (Fig. 6C). This led to a complete inhibition of the induction of antigen-specific proliferation. In contrast, 3-Deaza and MTA showed no toxic effects on T cells but still inhibited antigen-specific proliferation. Nevertheless, the inhibition was less pronounced compared to Aza-CdR and DZNep. We conclude that MTA exerts its effects through a mechanism different from interfering with DNA methylation or specific histone methylation.

It has been shown that MTA can interfere with asymmetric protein methylation upon T cell stimulation. To confirm that MTA exerted indeed an impact on global asymmetric methylation events, stimulated T cells were lysed after distinct periods of time and a Western blot was performed to detect global asymmetric protein methylation. Fig. 6D shows that global asymmetric protein methylation was affected by 500 μM MTA. There was a pronounced difference between the methylation status of T cells treated with MTA and untreated controls. The most prominent differences were observed for proteins of about 100 kDa. The global symmetric protein methylation was not affected (data not shown). Therefore, interference with asymmetric protein methylation events might explain the inhibitory effect of MTA on T cell activation.

Assessment of the secretion of MTA in MTAP-negative tumor cells and its impact on lymphocytes

To confirm the relevance of the suppressive effects of exogenously added MTA on human T cells, we used the MTAP-deficient melanoma cell line Mel-Juso that was stably transfected with the MTAP-coding sequence (Fig. 7A) and determined the effects of MTAP re-expression and endogenously secreted MTA on T cell proliferation. Analysis of MTA secretion by mass spectrometry revealed concentrations of 1 μM and 3 nM MTA in the supernatants of Mock- and MTAP-transfected melanoma cells, respectively, thus confirming the catabolism of MTA by MTAP. Expression of MHC-I and other surface molecules did not differ between Mock- and MTAP-transfected melanoma cells.

Figure 6. Mechanism of T cell inhibition. (A) Freshly isolated human CD8+ T cells were treated with 10 μM dipyridamole and stimulated with anti-CD3/CD28 beads in the presence or absence of 250 μM MTA and adenosine, respectively. After 48 h, cells were harvested, stained with anti-CD8 and CD25 antibodies and analyzed by flow cytometry (n = 3). (B) Determination of MTA and adenosine amounts by mass spectrometry in pure medium or in co-culture of freshly isolated human CD8+ T cells stimulated with moDCs pulsed with Mart-1 peptide on day 0, 2, 4, 6 and 8. (C) Freshly isolated human CD8+ T cells were stimulated with moDCs pulsed with Mart-1 peptide. Cells were cultured in medium supplemented with 100 U/mL IL-2. Different methylation inhibitors were added at a concentration of 25 μM to the culture: 5′-deoxy-5′-methylthioadenosine (MTA), 3′-deazaadenosine (3-Deaza), 5′-aza-2′-deoxycytidine (Aza-CdR) or 3′-deazaneplanocin A (DZNep). Left panel: Apoptotic T cells were identified by annexin-V/7-AAD staining. Numbers represent percent viable cells (annexin-V/7AAD) cells on day 11 (n = 3). Right panel: Frequency of Mart-1-specific CD8+ T cells was determined after 2nd stimulation (d11) by Mart-1 multimer staining. Numbers represent percent Mart-1+ peptide-specific CD8+ CTL (n = 3). (D) Freshly isolated human CD8+ T cells were stimulated with moDCs pulsed with Mart-1 or peptide in medium supplemented with 100 U/mL IL-2. After four rounds of stimulation, cells were harvested and incubated overnight without cytokines in the presence or absence of 500 μM MTA. Next, cells were stimulated with 20 ng/mL PMA and 1 μM ionomycin for the depicted periods of time or left unstimulated. Cells were lysed and lysates were used for protein gel blot analysis with an antibody specific for asymmetric arginine methylation (α-DMA). One representative experiment out of three is shown. Most prominent changes in asymmetric methylation patterns were observed in proteins of about 100 kDa size (red arrow). Quantification was done with ImageJ.
cells (data not shown). To analyze the effects of endogenously secreted MTA on T cells irradiated (100 Gy) MTAP- and Mock-transfected Mel-Juso melanoma cells were used as stimulator cells for allogeneic PBMCs in a mixed lymphocyte/tumor cell culture (MLTC). As shown in Fig. 7B, T cells stimulated with Mock-transfected melanoma cells (high MTA secretors) revealed a reduced proliferative capacity compared to T cells stimulated with MTAP-transfected melanoma cells (low MTA secretors). In addition, the secretion of MTA by the tumor cells influenced cytokine secretion by the PBMCs (Fig. S8). This result indicates that constant secretion of low amounts of MTA leads to anti-proliferative effects in T cells.

Discussion

Tumor antigen-specific T cells can be found in a broad variety of malignancies such as melanoma, renal cell carcinoma, breast and pancreatic adenocarcinoma. However, T cell infiltration is not always associated with a better prognosis. Even the promising approach of adoptive transfer of tumor-specific T cells against malignant cells does not consistently yield satisfying results. Several factors for inducing immune defects in T cells have been proposed, including the immunosuppressive setting of the tumor microenvironment. An emerging concept of the tumor microenvironment is the suppression of an adaptive immune response by tumor metabolites. In recent years, expression of MTAP in cancers has gained increasing interest. Because of the accumulation of MTA in the tumor microenvironment of MTAP-negative malignancies, we addressed the question whether MTA exerts inhibitory effects on T lymphocytes.

In vitro exposure of polyclonally stimulated CD8+ T cells with MTA led to a significant dose-dependent reduction of T cell proliferation without an immediate induction of apoptosis. Interestingly, our data demonstrated that this effect was reversible. Upon exposure to exogenous MTA, we observed a dose-dependent reduction in the secretion of IL-2 and IFN-γ by Mart-1-specific CTLs. Additionally, the cytolytic capacity, a key feature of CTLs, was hampered. This was not due to reduced expression of granzyme-B and perforin (data not shown), but to a diminished degranulation capacity. This seems to be the crucial function for tumor-infiltrating lymphocytes, as these defects are often found in tumors with poor prognosis.
The addition of low concentrations of MTA to a co-culture of Mart-1-pulsed moDCs and autologous CD8+ T cells completely abrogated the in vitro expansion of Mart-1-specific T cells. Of particular importance, T cell suppression is not indirectly mediated by suppression of mature antigen-presenting cells, as they exhibited no change in the expression of MHC, co-stimulatory as well as co-inhibitory molecules after co-culture with MTA (data not shown). Despite stimulation in the presence of IL-2, MTA-treated T cells did not up-regulate activation molecules such as CD25 and CD69 and maintained a naive CD45RO-CCR7high/CD27high phenotype. Interestingly, MTA mediated also the downregulation of CD71, the transferin-receptor, and CD98, a part of the large neutral amino acid transporter 1 (LAT1) (data not shown). The downregulation of these two molecules has been linked to total energy of T cells.67 Therefore, we analyzed central components of the T cell activation signaling cascade such as Erk, p38 and Akt. Data demonstrated that MTA inhibited phosphorylation of Akt and Erk, but it had no impact on the phosphorylation of p38. The signaling pathways downstream of Akt, which were also affected by MTA as shown in Fig. 5, lead to induction of genes responsible for cell proliferation and survival.68 In line with these data, the addition of MTA to already tumor antigen-stimulated CTLs revealed not only an induction of apoptosis, but also a strong reduction of Mart-1-specific CTLs in comparison to non-specific bystander-activated T cells. Induction of cell death in over-activated T cells by Akt inhibition has been already translated into the clinic as a therapeutic strategy against T cell leukemias.69 However, the used concentration of 500 µM MTA, which was needed to show these effects, indicates that the blockade of Akt by MTA can explain the inhibition of effector functions as shown in Fig. 2.

An important consideration is the question whether the MTA concentrations employed in our in vitro experiments are actually representative for the intratumoral microenvironment. Stevens et al.26 reported secreted levels of 140 nM for melanoma specimens, while Limm et al.27 referred to intracellular concentrations of up to 100 µM. In vitro exposure of human T cells with MTA revealed a dual effect of MTA: To strongly inhibit polyclonally stimulated T cells or to suppress CTL effector functions in short-term assays (4–5 h), high MTA concentrations (100–500 µM) were needed. In contrast, a magnitude lower concentration was sufficient to inhibit the induction of antigen-specific CTLs in a more physiological autologous moDC/T cell co-culture. Intriguingly, concentrations in excess of 25 µM induced apoptosis in highly stimulated CTLs. This point is of special interest, as T cells themselves are able to catabolize MTA with their own MTAP. To further address this issue, we aimed to verify the inhibitory effects of MTA in a tumor cell model. Limm et al.27 had shown, that it was possible to re-introduce MTAP into MTAP-deficient melanoma cell lines and, thereby, to reduce the secretion of MTA. We used the described cell lines, which were either MTAP- or Mock-transfected, in a mixed lymphocyte/tumor cell co-culture and observed a stronger proliferation of lymphocytes when MTAP was re-expressed in the melanoma cells. Interestingly, Mock-transfected melanoma cell lines secreted enough MTA to maintain a constant extracellular concentration of 1 µM, which proved sufficient to interfere with lymphocyte proliferation. This reveals the immunosuppressive effect of constant MTA-secretion by tumor cells at physiological levels and, therefore, the relevance of MTA in the tumor microenvironment, which is shown here for the very first time.

It is known that adenosine can inhibit T cell function including cellular proliferation, synthesis of IL-2 and pro-inflammatory cytokines and up-regulation of CD25.49,70 Therefore, we sought to exclude the possibility that it is the adenosine residue of MTA, which mediates the inhibitory effect of MTA. The addition of dipyridamole, which is an inhibitor of nucleoside transport, proved capable of abrogating adenosine-mediated, but not MTA-mediated T cell suppression. It is still unclear whether MTA diffuses or is carried by transporters into the cell. Along with these data, Izasa et al. noted that MTA excretion and incorporation into human lymphoma cells was not affected by dipyridamole and nitrobenzylthioinosin.71 Additionally, in line with published data,72 we were able to increase adenosine- but not MTA-mediated T cell suppression by inhibition of the adenosine-degrading enzyme adenosine deaminase.

As MTA is a potent protein methylation inhibitor,23,33,48 and there are several reports showing that protein methylation plays a so far underestimated role in T cell stimulation,73-76 we investigated whether other synthetic protein methylation inhibitors could mimic the impact of MTA on T cells. Indeed, 3′-deazaadenosine40 and adenosine-2,3-dialdehyde77 suppressed T cells in a similar manner (data not shown), i.e. inhibition of antigen-specific T cells, but no direct induction of apoptosis. In contrast, a DNA methylation inhibitor, 5′-aza-2′-deoxycytidine,52 efficiently induced apoptosis in T cells. Furthermore, we observed a decrease in asymmetric protein methylation events upon T cell stimulation in the presence of MTA. Not-methylated proteins were most abundant in the range of about 100 kDa. Blanchet et al.53 reported that Vav1, a protein of 97 kDa of the CD28 signaling pathway, has to be methylated to be functional upon T cell stimulation. This could be one of the early targets of MTA.

We show for the first time that tumor-derived MTA suppresses T cell functions. The suppression of T cell effector functions by MTA is most probably mediated by interfering with asymmetric protein methylation events upon T cell stimulation and a decreased phosphorylation of Akt.

Material and methods

Primary cells

Mononuclear cells (MNCs) collected from healthy volunteers’ leukapheresis products were separated by density gradient centrifugation (PAN-Biotec). Informed consent was obtained from patients according to the Declaration of Helsinki. The study was approved by the local ethics committee.

Media, chemicals, cytokines, peptides, and generation of dendritic cells

Cells were cultured in RPMI-1640 medium supplemented with L-glutamine, penicillin-streptomycin (all Invitrogen), sodium pyruvate, MEM vitamin-solution (all Pan Biotech) and 2-mercaptoethanol (Gibco Life Technology) plus 10 % human AB-serum (PAN Biotec) (complete RPMI). The chemicals
MTA, 3-Deza and adenosine, were purchased from Sigma-Aldrich, Aza-GdR was obtained from Biomol/Cayman. The HLA-A2-binding peptide Melan-A26-35 (ELAGIGILTV) was prepared by Bachem. Soluble recombinant HCMV pp65 (low endotoxin) was obtained from Miltenyi Biotec. Immature monocyte-derived dendritic cells (moDCs) were generated by incubating elutriated monocytes from HLA-A2+/C14 with 30 μg/mL MTA and 3-Deaza and adenosine, were purchased from Sigma-Aldrich. For phenotypic analysis, the following mAbs were used in medium plus 10% FCS (PAA) at 37°C and 5% CO2. Cells were passaged every 3 d. For CD8+ T cell assays, mature moDCs (2 x 10^6) were pulsed for 2 h at 37°C with 30 μg/mL MTA and 3-Deaza and adenosine, were purchased from Sigma-Aldrich. Subsequent maturation of immature moDCs was achieved by the addition of 10 ng/mL TNF, 1000 U/mL IL-6, 10 ng/mL IL-1β (all from Promokine) and 1 mg/mL PGE2 (Enzo Life Science) over a period of 3 d. For CD8+ T cell assays, mature moDCs (2 x 10^6) were pulsed for 2 h at 37°C with 30 μg/mL MTA and 3-Deaza and adenosine, were purchased from Sigma-Aldrich.

**Generation of antigen-specific CD8+ CTLs**

Mart-1-specific CD8+ cytotoxic T lymphocytes (CTLs) were generated as described previously. Briefly, CD8+ T cells were magnetically enriched (Miltenyi Biotec) from peripheral blood mononuclear cells (PBMC) of healthy donors and restimulated weekly with Mart-126-35 peptide-pulsed moDCs (generated as described above) in medium containing 100 U/mL IL-2 (Proleukin). Mart-1-specific T cells were determined by Mart-1-MHC tetramer staining (Beckman Coulter).

**Antigen-specific stimulation of CD4+ T cells**

CD4+ T cells were magnetically enriched (Miltenyi Biotec) from PBMCs of healthy donors and restimulated weekly with autologous moDCs that were pulsed with recombinant pp65 protein (as described above) in medium containing 10 U/mL IL-2 (Proleukin).

**Cell lines**

Human cell lines were cultured in supplemented RPMI-1640 medium plus 10% FCS (PAA) at 37°C and 5% CO2. Cells were passaged every 3–4 d. MTAP-deficient Mel-Juso cell lines were transfected with MTAP or Mock and treated as described elsewhere.

**Flow cytometry**

For phenotypic analysis, the following mAbs were used in accordance with manufacturer’s recommendation: α-CD3-PE, α-CD98-PE, α-CD25-PECy7, α-CD45R0-PECy7, α-CD8-PerCP, α-CD3-APC, α-CD38-APC, α-CD137-APC, α-CD8-APC-Cy7, α-CD3-APC-Ch, α-CD27-V450, α-CD127-V450, α-CCR7-V450, α-CD4-V500 (BD/PharMingen); α-CD71-FITC (Beckman Coulter); α-CD54-FITC (Acris); α-CD69-PE (Invitrogen) and α-CD107a-PE (eBioscience/NatuTec). Appropriate fluorochrome-labeled isotypes were used as controls. Intracellular FoxP3 was determined using the eBioscience FoxP3 Staining Buffer Set according to the manufacturer’s instructions and stained with α-FoxP3-APC (eBioscience). Prior to intracellular cytokine staining, CTLs were incubated for 5 h with autologous Mart-1-pulsed moDCs in the presence of MTA and monensin (BD Lifesciences). Cells were harvested, fixed, permeabilized, and stained with α-IL-2-PE and α-IFN-γ-FITC mAbs. Viability of T cells was determined by means of annexin-V-FITC and 7-AAD (BD Lifesciences) staining according to the manufacturer’s instructions. Phosphorylated intracellular signaling proteins were measured with the antibodies α-p38-MAPK-(pT180/pY182)-PE, α-Akt-(pS473)-PE or α-Erk1/2-(pT202/pY204) by intracellular staining using BD Phospho reagents, following the manufacturer’s instructions (BD Biosciences). Flow cytometric analyses were performed on a FACSCanto II (BD Biosciences). FACS data were analyzed with FlowJo software PC version 7.6.5 (Celeza).

**Proliferation assays**

Resting CD8+ or CD4+ T cells were magnetically enriched from PBMCs and stained with 4 μM CFSE (Fluka) for 4 min in the dark. After 5 d, cell division was measured by flow cytometry. CFSE+ T cells (2 x 10^5) were seeded in 225 μL/well in 96-well microtiter plates in the presence or absence of MTA at indicated concentrations. T cells were cultured with T cell expansion Dynabeads conjugated with activating anti-CD3 and anti-CD28 antibodies at a ratio of 2:1 (Invitrogen) in complete RPMI medium for 5 d. For cell cycle studies, cells were harvested after 48 h, fixed, permeabilized (BD), stained with Ki-67-PE (BD/PharMingen) or Cyclin E-FITC (Santa Cruz Biotechnologies) and further analyzed by flow cytometry.

**Cytokine detection**

To analyze the T helper cell profile, supernatants of CD4+ T cells/protein-pulsed moDCs co-cultures were collected after 24 h. Production of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF, lymphotoxin-α and IFN-γ was measured from the supernatants by means of a human TH1/TH2 Multiplex kit (eBioscience) according to the manufacturer’s instructions.

**Cytotoxicity assay**

CTL-mediated cytotoxicity was determined using a classical Chromium release assay as described previously. Percentage of specific cellular lysis was calculated with the following formula:

\[
\text{% specific lysis} = \frac{(\text{experimental cpm} - \text{basal cpm})}{(\text{maximal cpm} - \text{basal cpm})} \times 100
\]

Maximum ^51Cr release was determined by adding perchloric acid (3 % final concentration) to the target cells, while basal release was measured in the absence of effector cells.
PathScan® sandwich immunoassay

For simultaneous analysis of 18 significant, well-characterized intracellular signaling molecules, the PathScan® Intracellular Signaling Membrane Array Kit (Cell Signaling Technology) was used according to the manufacturer’s instructions. Briefly, equal amounts of cells were harvested and lysed in provided 1X Cell Lysis buffer. The membranes were blocked with Membrane Array Diluent Buffer for 1 h at RT. Then, lysates were added to each membrane and incubated overnight at 4°C on an orbital shaker. After several washing steps, membranes were incubated with 1x Detection Antibody Cocktail for 1 h at RT. Subsequent to further washing steps, 1x HRP-linked Streptavidin was added to each membrane for 30 min at RT. Finally, the membranes were washed again and treated with 1x Chemiluminescent Reagent. Images of the membrane arrays were acquired using the FluoroChem™ FC2 digital imaging system and array spot intensities were analyzed by ImageJ software.

cAMP ELISA

To assess cytosolic cAMP concentrations, T cells were washed three times in ice-cold PBS, lysed in 0.1 N HCl (107/mL) and a cAMP-specific ELISA (Correlate EIA Direct Cyclic AMP Enzyme Immunoassay kit; Assay Design) was performed.

Protein gel blot analysis

1 × 10⁶ cells were harvested and washed twice with PBS. Pellets were shock frozen in liquid nitrogen and stored at −80°C. Upon thawing, cells were lysed in Laemmli-buffer containing β-mercaptoethanol for 10 min at 95°C. Equal volumes of lysate were loaded, separated on 10% SDS-PAGE gels (PowerPac Basic, BioRad, Hercules, CA, USA) and subsequently blotted onto a PVDF membrane (35 min, 15 V, Trans-Blot Turbo, BioRad). After blocking for 1 h with 5% non-fat dry milk/PBS 0.1% Tween20 (Merck), the membrane was incubated for 16 h at 4°C with the primary antibodies against dimethyl-arginine, symmetric (SYM10) and asymmetric (ASYM24) (1:500; Millipore), Akt (40D4) (1:1000; Cell Signaling Technology), ERK1/2 (137F5) (1:1000; Cell Signaling Technology), GAPDH (ab9484) (1:10000; Abcam), β-actin (ab8224) (1:5000; Abcam) or MTAP (1:500, ProteinTech) in 2% non-fat dry milk/PBS 0.1% Tween20. The membrane was washed thrice in PBS-Tween, incubated for 1 h with a horseradish peroxidase-conjugated or alkaline phosphatase-conjugated secondary anti-rabbit antibody (1:5000, Cell Signaling Technology) or HRP-conjugated secondary anti-mouse antibody (1:5000; DAKO) in 2% non-fat dry milk/PBS 0.1% Tween20 and then washed again. Finally, immune reactions were visualized by Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare Europe GmbH) according to the manufacturer’s recommendations. Images were acquired with FluorChem FC2 (Cell Biosciences) and, quantified with ImageJ software (Rasbd WS).

MTA extraction and analysis by LC-ESI-MS/MS

Samples were prepared as described by Stevens et al.8¹,8² Briefly, for determination of extracellular MTA, 200 µL of cell culture medium were transferred to 2-mL Eppendorf tubes (Eppendorf) and spiked with 10 µL stable isotope labeled internal standard prior to protein precipitation with MeOH/0.1 M acetic acid (80:20, v/v). After centrifugation, the supernatant was transferred to a 1.5-mL glass vial (Fisher Scientific). The remaining protein pellet was washed twice with MeOH/0.1 M acetic acid (80:20, v/v) and the combined extracts were dried by means of an infrared evaporator. The residues were reconstituted in 100 µL of 0.1 M acetic acid.

Frozen cell pellets were spiked with 10 µL of internal standard. 600 µL of MeOH/0.1 M acetic acid (80:20, v/v) were added and cell pellets extracted by a freeze/thaw cycle (three times). After centrifugation, the supernatant was transferred to a 1.5-mL glass vial and the protein pellet was washed twice. The combined extracts were dried and reconstituted in 100 µL of 0.1 M acetic acid.

Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was carried out according to Stevens et al.8² on an Agilent 1200 SL HPLC system (Böblingen, Germany) coupled to a 4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany). An Atlantis T3 3 µm (2.1 mm i.d x 150 mm) reversed phase column (Waters, Eschborn, Germany) served as stationary phase, while the mobile phase contained 0.1% acetic acid and 0.025% heptafluorobutyric acid. Liquid chromatographic separation was carried out using a water-acetonitrile gradient at a flow-rate of 0.4 mL/min. Injection volumes were 10 µL. The mass spectrometer was operated in positive mode and quantitative determination of MTA was achieved by multiple reaction monitoring (MRM).

Statistics

For statistical analysis, the PrismGraph 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the Kolmogorov–Smirnov test. For Gaussian distributed groups, a paired or unpaired t-test was performed. For non-Gaussian distributed groups, the Mann–Whitney non-parametric U test (unpaired) or the Wilcoxon signed rank test (paired) was performed. The p-values are indicated as *p < 0.05, **p < 0.01 and ***p < 0.001. If not otherwise stated, data are shown as means ± SEM.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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