Regulatory T cells reduce endothelial neutral sphingomyelinase 2 to prevent T-cell migration into tumors

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Endothelial cells are key regulators of transendothelial migration and their secretion of chemokines and expression of adhesion molecules facilitates lymphocyte entry into tissues. Previously, we demonstrated that Tregs can reduce transendothelial migration of T cells into tumors by decreasing endothelial CXCL10 secretion, but the mechanism by which this occurs is still not known. In this study, we aimed to define how Tregs decrease transendothelial migration into tumors. mRNA sequencing of intestinal tumor endothelial cells from Treg depleted mice identified neutral sphingomyelinase 2 (nSMase2) as a gene downregulated in the presence of Tregs. nSMase2 is expressed in human umbilical vein endothelial cells (HUVECs) and was decreased after coculture with Tregs. Furthermore, blocking of nSMase2 activity in vitro decreased VCAM1, CX3CL1, and CXCL10 expression in HUVECs, mirroring the same decrease found in Treg cocultures. In the APCmin/+ mouse model of intestinal cancer, nSMase2 is lower in tumor endothelial cells than in unaffected small intestine and chronic treatment with a nSMase2 inhibitor suppressed the increased migration that is otherwise seen in the absence of Tregs. We conclude that nSMase2 is an important mediator in endothelial cells supporting transendothelial migration, which may be targeted by Tregs to reduce T-cell migration into tumors.

Keywords: CXCL10 · endothelial cell · nSMase2 · regulatory T cells · VCAM-1

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Effective tumor immunity is dependent on activated effector lymphocytes, both cytotoxic CD8+ T cells and NK cells, but also Th1
cells secreting cytokines to support differentiation and activity of cytotoxic cells [1]. These cells need to leave the blood stream and migrate into the tumor, in order to properly execute their antitumor effects. Endothelial cells are key regulators of lymphocyte transmigration from blood into tissue, and their expression of adhesion molecules and chemokines regulate lymphocyte influx into the tumor [2]. The composition of lymphocyte subsets in the tumor tissue is crucial for patient outcome. This notion has been thoroughly validated in colorectal cancer (CRC), where the so-called immunoscore index, defining number and location of CD3+ and CD8+ cells in tumors, is more accurate than established prognostic factors, tumor stage or microsatellite status, for predicting disease outcome and response to treatment [3, 4]. In addition, the balance between Th1 and Th17 cells contributes to patient outcome in CRC, where a pronounced Th1 infiltration correlates to a positive patient outcome, while Th17 infiltration correlates to a negative outcome [5]. Furthermore, a large proportion of Tregs in the immune cell infiltrate in solid tumors correlates to a poor patient outcome [6, 7]. Together, these findings indicate the importance of a balanced immune response to prevent tumor progression.

Recently, checkpoint blockade therapy has led to a paradigm shift in cancer treatment [8]. However, it is only successful in a fraction of patients, and in CRC this is mainly in the small subgroup with high microsatellite instability tumors [9–11]. Therefore, alternative treatment options are highly warranted, and targeting Treg activity might be one potential way forward. Depletion of Tregs leads to devastating autoimmunity [12], and therefore, more sophisticated approaches are needed, aiming at only one or a few Treg functions, preferably only in the tumor microenvironment.

Additionally, Tregs accumulate in CRC [6, 13–15] and contribute to an immunosuppressive tumor microenvironment. Tregs can reduce proliferation and effector functions in both CD4+ and CD8+ T cells [16]. Furthermore, depletion of Tregs leads to increased T-cell accumulation in several tumor models [12, 17–20], due to both increased infiltration and proliferation [21]. We have previously shown that T-cell migration into tumors is dependent on CXCR3-mediated signaling in the APCmin/+ mouse model of intestinal tumors and that Treg depletion increases CXCL10 production in blood vessel endothelial cells in the tumors [17]. Previous in vitro experiments also show that Tregs substantially inhibit T-cell transendothelial migration, either directly through TGFβ-secretion or indirectly via the generation of adenosine [22, 23]. However, the mechanisms used by Tregs to prevent T-cell recruitment in vivo are still not established.

Here, we used mRNA sequencing in tumor endothelial cells from Treg depleted APCmin/+ mice as an unbiased approach to identify endothelial cell mediators that are modulated by Tregs in tumors. We found that neutral sphingomyelinase 2 (nSMase2), a mediator of TNF-induced endothelial activation, is downregulated in the presence of Tregs. Tregs suppress endothelial nSMase2 expression in vitro, as well as endothelial adhesion molecules and chemokines, and the effect of Tregs is mimicked by pharmacologically blocking nSMase2 activity. In vivo, nSMase2 inhibition also blocks the increase in T-cell migration to tumors induced by Treg depletion.

**Results**

**Several genes in tumor endothelial cells are affected by the presence of Tregs**

T-cell migration into tumor tissue is crucial for an antitumor response. Previously, we had demonstrated that Tregs decrease the numbers of conventional T cells in intestinal tumors partly by reducing the expression of the CXCR3 ligand CXCL10 specifically in tumor endothelial cells [17, 21]. In order to get a broad and unbiased view of how Tregs affect tumor endothelial cells and reduce transendothelial migration, we sought to find endothelial genes that were influenced by Treg presence and that could explain decreased migration into tumors. Thus, intestinal tumor blood vessel endothelial cells from Treg depleted and untreated control mice were sorted, and mRNA sequenced in order to identify differentially expressed genes. In total, 450 significantly upregulated and 378 significantly downregulated genes were found in endothelial cells from Treg depleted mice compared to Treg sufficient mice. Supporting information Table S1 shows the 15 upregulated genes with lowest false discovery rate adjusted p values, where Smpd3 was one of the genes with an over twofold increased expression in the absence of Treg. Smpd3 is the gene for nSMase2, an enzyme that generates the bioactive lipid ceramide through hydrolysis of the membrane lipid sphingomyelin and which has also been implicated in tumor progression and migration of tumor cells [24–28].

**nSMase2 is expressed in mouse and human tumor endothelial cells**

To validate the sequencing data, nSMase2 was first investigated in mouse and human intestinal tumor endothelial cells. In mouse unaffected intestine and tumors, nSMase2 protein was detected both in normal blood vessel endothelial and lymphatic endothelial cells (Fig. 1A). A significant reduction of blood vessel endothelial nSMase2 expression in the tumors compared to unaffected tissue was also recorded (Fig. 1A). Additionally, nSMase2 was expressed by endothelial cells from human colon tumors, however, no difference between unaffected colon tissue and tumor tissue was detected (Fig. 1B). Furthermore, in primary human endothelial cells, nSMase2 was consistently detected, and following 24-h stimulation with TNF, there was an increased expression in both mRNA and protein levels (Fig. 1C). Together, these findings show that nSMase2 is consistently expressed in intestinal blood vessel endothelial cells across species.
Figure 1. nSMase2 expression in tumor endothelial cells. (A) nSMase2 expression was analyzed by flow cytometry in mouse endothelial cells isolated from small intestine (SI) and tumors of APC<sup>min/+</sup> mice. Histogram shows one representative mouse and graph shows nSMase2 expression in blood vessel endothelium from unaffected tissue and tumor in APC<sup>min/+</sup> mice. Data are from two experiments with two mice per experiment. (B) nSMase2 expression in blood vessel endothelial cells isolated from human colon cancer patients and analyzed by flow cytometry. Histogram shows one representative patient and the graph shows endothelial nSMase2 expression in tumor and unaffected tissue data from seven experiments with seven patients in total. (C) nSMase2 mRNA normalized against 18s and protein expression in HUVECs treated with TNF for 24 h (n = 5-10, data from three experiments) by qPCR and flow cytometry, respectively. Line represent mean and error bar SD. *p < 0.05, **p < 0.01; Unpaired t-test.

Figure 2. Tregs reduce nSMase2 expression. (A) Whole small intestine and intestinal tumor nSMase2 mRNA levels determined by RT-PCR normalized against β-catenin, in APC<sup>min/+</sup> mice with or without Tregs. n = 6-10, data from three experiments. Line represent mean, **p < 0.01; unpaired t-test. (B) nSMase2 protein expression in HUVECs after 6 h of coculture with or without Tregs. Histogram shows one representative experiment and graph display relative nSMase2 MFI values from six experiments with one sample per experiment, **p < 0.01; paired t-test.

Tregs reduce the endothelial expression of nSMase2

As nSMase2 expression was influenced by the presence of Tregs in mRNA sequencing experiments, we sought to confirm this with RT-PCR in whole tumor tissue from Treg depleted mice. Tumors derived from Treg depleted mice showed a significant increase of nSMase2 expression compared to Treg sufficient tumors (Fig. 2A). Notably, the effect was only detected in tumor tissue, and not in the unaffected small intestine. However, to establish if Tregs have a direct effect specifically on endothelial cells, we created a coculture system where sorted CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs from PBMC were cultured together with HUVECs. After 6 h of coculture, we could detect a significant decrease in nSMase2 in the endothelial cells (Fig. 2B), confirming a direct effect of
Tregs on endothelial expression of nSMase2. On average, a 1.2-fold decrease in nSMase2 MFI was detected using Tregs from six different donors. The different culture conditions did not affect Treg activation stage, as judged by expression of activation markers (CD69, CD25, PD-1, CD38, and HLA-DR) on Treg, when cultured in the presence or absence of HUVEC, TNF, or DP-TIP (n = 3 different donors).

nSMase2 promotes expression of adhesion molecules and chemokines in endothelial cells

Next, we wanted to assess the contribution of nSMase2 on the ability of endothelial cells to recruit T cells. Stimulation of endothelial cells by proinflammatory cytokines is an important way to increase immune cell recruitment into tissues. We used 2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol (DPTIP), a newly identified potent and specific nSMase2 inhibitor in vitro [29] to investigate the contribution of nSMase2 in this process. In TNF-stimulated HUVECs, DPTIP decreased not only nSMase2 activity, but also nSMase2 protein expression (Fig. 3A). HUVECs were pretreated with DPTIP and subsequently stimulated with TNF for 4 h before Nanostring analyses of mRNA production. nSMase2 inhibition resulted in a significant decrease in several mRNAs coding for proteins of importance for transendothelial migration (Fig. 3B), among them VCAM1, CXCL11, CX3CL1, and CXCL10. These results were subsequently confirmed with RT-PCR analyses, where a significant decrease in VCAM1, CXCL10, and CX3CL1 mRNA, but not ICAM1 or CXCL9 mRNA, was detected after nSMase2 inhibition in HUVECs (Fig. 3C). Furthermore, HMVECs showed a similar decrease in VCAM1 and CXCL10 mRNA when nSMase2 was inhibited before TNF stimulation (Supporting information Fig. S1). To confirm the mRNA data, protein expression was investigated in HUVECs by flow cytometry after 4 or 24 h of stimulation with TNF with or without prior nSMase2 inhibition. VCAM1, CXCL10, and CX3CL1 expression were rapidly decreased with nSMase2 blocking, and the lower levels were sustained at 24 h for VCAM1 and CXCL10. In contrast, neither ICAM1, nor CXCL9 protein expression was affected by nSMase2 inhibition (Fig. 3D). To investigate if nSMase2 inhibition only affects TNF-induced changes in endothelial cells, HUVECs were stimulated with IL-1ß, LPS, or IFN-ß (Supporting information Fig. S2). These stimulations did not change nSMase2 expression, and expression of nSMase2 was also unchanged after DPTIP treatment, regardless of stimulation (data not shown). IL-1ß induced increased expression of VCAM1 and ICAM1 already after 4 h, while IFN-ß stimulation led to increased expression of ICAM1 and CXCL9 after 24 h. Blocking of nSMase2 prior to stimulation with either IFN-ß or LPS did not result in any changes in expression of the investigated molecules. However, IL-1ß-induced expression of VCAM1, but not CXCL10, was dependent on nSMase2 activity (Supporting information Fig. S2).

Taken together, these data confirm that nSMase2 controls the expression of specific adhesion molecules like VCAM1, but also adds new molecules to the list of mediators controlled by nSMase2 activity in endothelial cells following TNF stimulation.

Tregs directly influence the expression of adhesion molecules and chemokines regulated by nSMase2

Since Tregs can downregulate nSMase2 expression in endothelial cells, we also wanted to confirm that Tregs directly reduce endothelial markers dependent on nSMase2. After coculture with sorted Tregs, HUVECs showed significantly decreased expression of VCAM1, ICAM1, ICAM2, CXCL10, and CX3CL1, while E-selectin and CXCL9 were unchanged (Fig. 4A). The largest effect was seen in VCAM1 and ICAM1, while changes in ICAM2, CXCL10, and CX3CL1 were more modest. The reduced ICAM1 expression in these experiments may indicate that Tregs can reduce ICAM1 through other means than nSMase2 signaling. We also examined the release of chemokines and soluble forms of VCAM-1 and ICAM-1 into the culture medium. The chemokines were barely detected, presumably because we used Golgistop to prevent secretion in order to optimize flow cytometry detection. On the other hand, TNF stimulation increased the concentration of both soluble ICAM-1 and VCAM-1 in the cultures, and the concentrations were reduced on addition of Treg (Supporting information Fig. S3).

In order to understand the mechanisms behind Treg suppression of endothelial adhesion molecule expression, we compared the effect of coculture with Tregs to that of conditioned medium from the same Tregs, and found a consistent suppression of ICAM1, VCAM1, and CXCL10 (Fig. 4B) with the conditioned medium, similar to that seen after coculture with Tregs. This coincided with a decreased expression of nSMase2 after addition of Treg conditioned media (Supporting information Fig. S4). These observations suggest that soluble factors from Tregs can suppress endothelial expression of nSMase2 leading to suppressed expression of adhesion molecules and chemokines. Maganto-Garcia et al. has previously proposed that Tregs downregulate VCAM1 expression by secretion of TGF-ß [23], and we, therefore, blocked TGF-ß in the conditioned media before adding it to HUVECs. Blocking of TGF-ß in Treg conditioned media with latency-associated peptide (LAP) partly abrogated the suppression of ICAM1, VCAM1, and CXCL10 seen with untreated conditioned media (Fig. 4C). However, treatment of HUVECs with TGF-ß alone did not decrease the expression of adhesion molecules and chemokines (data not shown). In contrast, blocking of IL-10 with a neutralizing antibody did not affect VCAM-1 and ICAM-1 expression in HUVEC (Supporting information Fig. S5). These experiments demonstrate that Tregs directly affect molecules important for transendothelial migration by secreted products, and that TGF-ß appears to contribute to the Treg effects on endothelial cells. Furthermore, nSMase2 inhibition mimics the effect of Tregs on endothelial cells with regard to the modulation of endothelial protein expression.
Figure 3. The nSMase2 inhibitor DPTIP decreases expression of several genes important for transendothelial migration. HUVECs were either pre-treated 1 h with DPTIP or left untouched, followed by 4 or 24 h of TNF stimulation. (A) Expression of nSMase2 determined by flow cytometry, showing one representative histogram and a graph showing relative MFI values. Data points show the mean value of biological triplicates from five experiments, one sample per experiment. (B) Nanostring gene counts presented in a heat map showing TNF-stimulated HUVECs treated with DPTIP compared to TNF stimulation alone. (C) VCAM1, CXCL10, CXCL11, ICAM1, and CXCL9 mRNA levels determined by RT-PCR normalized against 18s in HUVECs stimulated with TNF for 4 h, and either pretreated with DPTIP or left untreated (n = 4-6, data from two experiments). Bars represent mean and error bars SD. (D) Protein expression of VCAM1, CXCL10, CXCL1, ICAM1, and CXCL9 detected by flow cytometry in HUVECs stimulated with TNF and either pretreated with DPTIP or left untreated for respective 24 h. Histograms show representative staining of HUVEC cells at 4 h, vertical lines represent the FMO cut-off for positive staining. Symbols represent individual values and horizontal lines the mean, n = 6-15, data from six experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Unpaired t-test.
nSMase2 is needed to sustain TNF signaling through TNFR1

nSMase2 is a component of the TNFR1 complex during receptor signaling [30] leading to adhesion molecule expression. On the other hand, CXCL10 production after TNF stimulation is induced by an Interferon regulatory factor 1 (IRF1)-dependent autocrine loop in endothelial cells, comprising TNFR1, IRF1, INF-β, IFNAR, and STAT1 [31, 32]. We, therefore, investigated the expression of the components of this signaling cascade when nSMase2 activity was blocked. We showed that several components in this loop were decreased upon nSMase2 inhibition in HUVECs (Supporting information Fig. S6), indicating that nSMase2 is indeed needed to sustain expression of CXCL10 through TNFR1. Therefore, these experiments show that nSMase2 contributes to TNF signaling in endothelial cells, not only by ceramide generation but also through IRF1 and subsequent downstream autocrine signaling.
Tumor endothelial cell expression of adhesion molecules

Blood vessel endothelial cells from human colon cancer tissue were investigated for their expression of the markers identified in vitro as dependent on nSMase2. Previously, we had seen reduced levels of CXCL10, but not CXCL9, expression in tumor endothelial cells compared to unaffected tissue [17]. Here, we detected a significant reduction of endothelial ICAM1 expression in tumors, while ICAM2, VCAM1, and CX3CL1 were similar in endothelial cells from tumors and unaffected tissue (Supporting information Fig. S7A). However, the expression of VCAM1 was low in endothelial cells from both unaffected and tumor tissue compared to HUVECs. We also tried to correlate nSMase2 expression with adhesion molecule expression in individual cells from tumors. In these analyses, the VCAM1+ endothelial cells indeed had a higher nSMase2 expression than the VCAM1− endothelial cells (Supporting information Fig. S7B).

Molecules that were dependent on nSMase2 for optimal expression were also examined in APCmin/+ tumors. VCAM1 and CX3CL1 expression were significantly lower in endothelial cells from APCmin/+ tumors than unaffected intestine (Supporting information Fig. S8). Furthermore, the Treg depletion regime did not alter VCAM1, ICAM1, and CX3CL1 expression (data not shown).

nSMase2 activity improves T-cell migration into tumors

Since nSMase2 controls adhesion molecules important for lymphocyte migration, we speculated that nSMase2 activity could influence transendothelial migration into tumors. We have previously shown that Treg depletion leads to increased lymphocyte accumulation in tumors of APCmin/+ mice, due to increased migration of T cells, but also increased intratumoral T-cell proliferation [21]. Based on our current results, we hypothesized that Treg depletion would lead to increased nSMase2 activity, and subsequent T-cell accumulation. In order to investigate if nSMase2 inhibition can counteract the effect of Treg depletion in vivo, APCmin/+DEREG mice were injected with diphtheria toxin (DT) in order to remove Tregs and simultaneously treated with phenyl (R)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-b]pyridazin-8-yl)pyrrolidin-3-yl)carbamate (PDDC), an nSMase2 inhibitor developed for in vivo use [29]. As controls, Treg depleted mice treated with the vehicle alone and Treg sufficient mice were used. We also investigated if PDDC treatment as such affected activation of conventional CD4+ and CD8+ T cells in vivo. These experiments showed that there was an increase in MHC class II on CD8+ T cells from MLN in the PDDC-treated animals, but not any other cells, and the expression of all other markers (CD25, CD69, PD-1, and ICOS) by both CD4+ and CD8+ cells from spleen and MLN was not changed. Furthermore, the frequencies of Treg, CD4+ non-Treg, and CD8+ T cells were unchanged in the spleen and MLN after the treatment period (n = 3 mice in each group). In addition, there was no effect on vessel density or morphology in the mice treated with PDDC. As previously observed [21], Treg depletion increased the migration of CD4+ T cells into tumors. However, Treg depleted mice treated with PDDC had a significantly lower migration of CD4+ T cells into tumors, indicating that nSMase2 activity is needed to facilitate increased transendothelial migration when Tregs are depleted (Fig. 5A). It is interesting to note, that although PDDC dampened the increased migration of T cells when Tregs were depleted, nSMase2 inhibition did not affect the increased T-cell proliferation resulting from Treg depletion (Fig. 5B). The VCAM1 ligand integrin α4β1 (CD49d) was more highly expressed on CD4+ cells infiltrating tumors in Treg depleted compared to untreated mice. However, such an increase was not observed in CD4+ cells from PDDC-treated mice. On the other hand, the T-cell expression of the receptors for CXCL10 and CX3CL1, CXCR3 and CX3CR1, was unchanged following both Treg depletion and nSMase2 inhibition (Fig. 5C). Taken together, nSMase2 activity in endothelial cells is important for lymphocyte migration into tumor tissue, and appears to be modulated upon Treg depletion.

Discussion

Tregs accumulate in intestinal tumors of both mice and humans [13–15, 33, 34] and reduces transendothelial migration of T cells into tumors [17, 21], thereby preventing an effective antitumor immune response. However, the process by which this occurs is still poorly characterized. In this study, we propose a mechanism where secreted factors from Tregs downregulate nSMase2 expression in endothelial cells, leading to reduced expression of adhesion molecules and chemokines needed for transendothelial migration.

Four different nSMases have been identified, but nSMase2 is the most predominant in cellular systems and pathologic conditions [35]. Upon ligand binding, nSMase2 forms a complex with TNFR1 leading to the hydrolysis of sphingomyelin into ceramide, which is a bioactive sphingolipid that regulates differentiation, cell cycle arrest, senescence, apoptosis, and autophagy [36]. Ceramide can be further hydrolyzed into sphingosine and sphingosine-1-phosphate (SIP). SIP has been implicated in endothelial barrier function and can regulate the endothelial expression of VCAM1 and ICAM1 [37] as well as certain chemokines [31, 38]. Here, we show that nSMase2 is expressed in tumor endothelial cells of both human and mouse origin, and also in HUVECs. Furthermore, we show that not only enzyme activity, but also enzyme mRNA and protein expression is upregulated upon TNF stimulation of endothelial cells.

Our results also demonstrate that presence of Tregs decreases the expression of nSMase2 in endothelial cells. This was first demonstrated by mRNA sequencing of tumor endothelial cells from APCmin/+ mice with or without Tregs. A direct effect of Tregs on endothelial nSMase2 expression was then confirmed in vitro, when HUVECs were cocultured together with Tregs. Our results indicate that similar mechanisms are active in both
A) CD4+CD25- and CD8+ cells in tumors and SI of Treg depleted and PDDC-treated mice. (B) Ki67 expression among CD4+ and CD8+ cells in tumors and SI of Treg depleted and PDDC-treated mice. (C) Expression of CD49d, CXCR3, and CX3CR1 by CD4+CD25- cells in tumors of Treg depleted and PDDC-treated mice. (A-C) n = 3-7 mice, data from four experiments. Line represent mean. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; Unpaired t-test.

Figure 5. nSMase2 is needed for lymphocyte migration into tumors. The nSMase2 inhibitor PDDC was administered to mice during Treg depletion and as control, Treg depleted and Treg sufficient mice were given vehicle chow. After 12 days, small intestines (SI) and their tumors were harvested and single-cell suspensions were prepared by enzymatic digestion and evaluated by flow cytometry for their composition of lymphocytes and expression of surface markers. Human and murine endothelial cells, and that the APCMin/+ mouse model may, thus, be a valid model of human CRC in this regard. In addition to the effect on nSMase2 expression, there is also a direct effect of Tregs on endothelial expression of adhesion molecules and chemokines, which can be mimicked by treating endothelial cells with a selective nSMase2 inhibitor. Both Tregs and the nSMase2-specific inhibitor DPTIP resulted in decreased expression of VCAM1, CX3CL1, and CXCL10 in HUVECs on both mRNA and protein level. nSMase2 activity and sphingomyelin conversion to ceramide is the first step towards generation of S1P [39], and S1P induces endothelial VCAM1 expression [37]. Our finding that Tregs decrease VCAM1, CX3CL1, and CXCL10 is compatible with a model where Tregs reduce nSMase2 activity to modulate endothelial recruitment of lymphocytes. However, ICAM1 expression was also affected by the presence of Tregs, but not after nSMase2 inhibition. ICAM1 is reported to be induced by S1P [37], but in the current study we could not detect a difference in ICAM1 expression after nSMase2 inhibition, which might indicate that Tregs use different pathways to regulate VCAM1 and chemokines on the one hand, and ICAM1 on the other. The factor(s) from Tregs that may confer the effect on nSMase2 are so far unknown, but in vitro, both adenosine and TGF-β have been proposed to reduce transendothelial migration [22, 23]. As our studies showed that soluble factors from Treg cultures were able to decrease VCAM1, ICAM1, and CXCL10 in HUVECs, adenosine, which has a very short half-life, is probably not a main mediator of the effect. Blocking of TGF-β restored some of the expression of VCAM1, CXCL10, and ICAM1. However, as recombinant TGF-β alone did not suppress expression of adhesion molecules and chemokines, suppression is probably mediated by the combined effect of several secreted products from Tregs, one of which is TGF-β.

It is interesting to note that several of the molecules affected by Tregs contribute to recruitment of Th1 and CD8+ T cells, not
least CXCL10 and CX3CL1. We have previously shown that lymphocyte migration into tumors is dependent on CXCR3 expression on T cells [17]. In the current study, we could complement this observation by showing that expression of the CXCR3 ligand CXCL10 is dependent on nSMase2 activity. In addition to autocrine signaling through cell surface S1P receptors, S1P has intracellular targets and can induce an IRF1 autocrine loop [39] which has previously been shown to induce increased CXCL10 secretion [31]. Several of the components of the IRF1 autocrine loop is decreased after nSMase2 blocking, showing that not only ceramide formation but also other signaling cascades contribute to the effects of nSMase2 on endothelial cells. In addition to CXCL10, we could record a significant decrease in endothelial production of CX3CL1, another chemokine that recruits CD8+ T cells and NK cells [40], following Treg culture and nSMase2 inhibition.

In mouse intestinal tumors, there was a decreased endothelial expression of nSMase2 compared to unaffected tissue, as well as a significant decrease in both VCAM1 and CX3CL1, possibly an effect of the high Treg frequencies in tumors [13–15]. In these studies, we chose to use small intestinal tumors from the APC<sup>Min/+</sup> mice, in order to achieve enough cells for mRNA sequencing and T-cell migration experiments. There are certainly large differences between small and large intestine, not least in morphology and microflora, but our previous study has shown similar composition of T cells in tumors in the two locations, and similar mechanisms for T-cell recruitment [21]. In this system, short-term depletion of Tregs had limited effect on the expression of CX3CL1 and ICAM1, and VCAM1 was unchanged. However, it is possible that the effects of Treg depletion are transient and not detected in our evaluation after 12 days. In patient colon tumors, no difference in endothelial nSMase2 expression was detected between normal and tumor tissue and no difference in VCAM1 or CX3CL1 expression was detected either. Nevertheless, we found a clear correlation between nSMase2 and VCAM1 expression in single endothelial cells, indicating the importance of nSMase2 activity for VCAM1 expression. ICAM1 on the other hand, was significantly decreased in patient tumor tissue, and probably additional mechanisms besides nSMase2 activity are present to regulate adhesion molecules on the tumor blood vessels.

We have previously shown that Treg depletion results in improved migration of conventional T cells into tumors [21]. In the current study, we could show that blocking of nSMase2 activity prevented the increased accumulation of CD4<sup>+</sup> T cells seen after Treg depletion. These experiments indicate that Treg act via reduced endothelial nSMase2 activity also in vivo, and use this effect to prevent T-cell accumulation in tumors. Our previous study also demonstrated that the increased accumulation of lymphocytes in tumors after Treg depletion is due to both increased migration but also increased T-cell proliferation [21]. Even though migration of T cells was decreased when nSMase2 activity was blocked, there was no effect on the proliferation of T cells, which was still higher than in Treg proficient mice. This observation indicates that Treg reduces nSMase2 activity to prevent T-cell migration into tumors, but that Treg inhibition of T-cell proliferation is mediated through a different mechanism. This may explain why nSMase2 inhibition does not completely prevent T-cell accumulation in the tumors following Treg depletion.

In the last few years, checkpoint blockade immunotherapy has improved the long-term survival for patients with malignant melanoma, non-small cell lung cancer, and other tumors with high mutational burden dramatically [9]. In CRC, on the other hand, it is primarily patients with high microsatellite instability tumors who respond to checkpoint blockade [10, 11]. As they make up only a small fraction of all CRC patients, novel treatments are urgently needed. At the same time, it is clear that lymphocyte infiltration into tumors is an important factor for patient survival [3, 4], and therefore, treatment options that improve this aspect of tumor immunity are likely to be successful. Therefore, targeting of intratumoral Treg or promotion of nSMase2 activity may be future treatment leads worth pursuing.

Taken together, endothelial nSMase2 controls several molecules important for lymphocyte migration, possibly through sphingolipid hydrolysis and the IRF1 autocrine loop. This study shows for the first time the crucial role of nSMase2 for lymphocyte migration into tumors and that soluble factors from Tregs directly reduce nSMase2-induced adhesion molecules and chemokines in endothelial cells. We suggest that Tregs block the nSMase2-S1P pathway in order to reduce transendothelial migration of effector T cells into tumors. Our results indicate that modulation of the Treg-endothelial signaling axis mediated by nSMase2 activity may provide a novel target for immunotherapy against intestinal tumors.

**Materials and methods**

**Mouse strains and breeding**

APC<sup>min/+</sup> mice, on a C57BL/6 background [41] and DEREG mice, expressing the DT receptor under control of the FoxP3 promoter [42], were bred to generate APC<sup>min/+</sup>/DEREG mice and APC<sup>min/+</sup> mice [21] at the Department of Experimental Biomedicine, University of Gothenburg. Four weeks after birth, APC<sup>min/+</sup> genotype was examined by PCR and DEREG phenotype by flow cytometry as previously described [41, 42]. Animals were kept under specific pathogen-free conditions in filter top cages.

Tregs were depleted in both female and male 18-week-old APC<sup>min/+</sup>/DEREG mice by i.p injections of 0.5 μg DT on day 1, 2, 8, and 9. As controls, APC<sup>min/+</sup> mice were identically treated. In some experiments, APC<sup>min/+</sup> or APC<sup>min/+</sup>/DEREG mice injected with DT were fed chow containing 100 mg drug/kg body weight of the specific nSMase2 inhibitor phenyl (R)-(1-(3-(3,4-dimethoxyphenyl)-2,6-dimethylimidazo[1,2-b]pyridazin-8-yl)pyrrolidin-3-yl)carbamate (PDDC) [43] or vehicle chow in order to block nSMase2 activity. One week prior to the start of DT, mice were put on vehicle chow and on the day of first injection
PDDC-containing chow was introduced. PDDC chow was changed every second day and on day 6 and 7 only vehicle chow was given. Mice were sacrificed and the intestine harvested on day 12, that is, at the age of 20 weeks. Lamina propria cells from small intestine and tumors were isolated as previously described, with either collagenase P for endothelial cell preparation or collagenase D for lymphocyte cell preparation [34, 44].

DPTIP and PDDC-containing chow

DPTIP and PDDC were synthesized as previously described [29, 43, 45]. In order to dose the mice with PDDC in an unstressful manner, PDDC was formulated into an OpenStandard Diet with 15 kcal% mouse chow at a dose that equates to 50 mg/kg daily with normal mouse consumption by Research Diets (New Brunswick, NJ). This dose provides sustained plasma PDDC levels which remain above its IC50 for nSMase2 inhibition for 24 h (personal communication with B Slusher).

Patient tissue

Colon cancer patients scheduled for curative surgery of primary stage II or III colon adenocarcinoma at Sahlgrenska University Hospital, Gothenburg, Sweden gave written consent to donate a piece of colon tumor and unaffected colon mucosa at the time of surgery. None of the patients had undergone radiotherapy or chemotherapy for at least 3 years prior to colectomy, and none suffered from autoimmune diseases. Tissue specimens were transported on ice and immediately used for cell isolation as previously described [46].

Primary endothelial cell lines

Human umbilical vein endothelial cells (HUVEC; Cascade Biologics Inc.) and human microvascular endothelial cells (HMVEC; Cascade Biologics Inc.) at passage 4-6 were seeded at 5 × 10^4 cells/well in M200 medium (Gibco) supplemented with LSGS kit (Gibco) in 24-well culture plates and cultured for 48 h followed by stimulation with 50 ng/mL of TNF (Biologend) for 4 or 24 h or left untreated. In separate experiments, cells were stimulated with either 1 ng/mL of IFN-γ (Biologend), 2 μg/mL of LPS (Sigma), or 10 ng/mL of IL-1β (Biologend), 10 ng/mL of TGF-β (Sigma) for 4 or 24 h. Pretreatment for 1 h with 50 μM of the specific nSMase2 inhibitor 2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol (DPTIP) (Biotechne) was used to block nSMase2 activity [29]. For detection of chemokines, Golgistop (BD) was added during the last 3 h of culture. Cells were harvested by trypsination and either analyzed by flow cytometry or frozen in lysis buffer RLT (Qiagen) for RT-PCR or Nanostring analyses.

mRNA sequencing

Blood vessel endothelial cells, identified as CD45– EpCAM– CD31+ Podoplanin+ cells, from DT-treated APCmin/+ and APCmin−/−/DEREG intestinal tumors were sorted on an Aria II flow cytometer (BD Biosciences) and immediately frozen in RLT buffer (QIAGEN) for further RNA isolation (see Supporting information Fig. S9A for gating strategy).

Total RNA was isolated as previously described [34] and amplified using SMARTer Pico from Clontech (Takara), followed by sequenced using a NGI RNA-seq analysis pipeline with Illumina sequencing at Science for Life Laboratory, Stockholm, Sweden. Resulting reads were mapped against the mouse genome and summarized on gene level. Only samples in which more than 50% of the reads mapped to the genome were included in the differential expression analysis, which was performed using the Bioconductor R package limma 3.42.2 [47] and analyzed accordingly to Law et al. [48]. Genes were considered significantly differentially expressed if they exhibited a false discovery rate adjusted p value below 0.05.

Treg coculture with endothelial cells

PBMC from healthy volunteers were isolated from buffy coats by Ficoll–Paque (Pharmacia) density-gradient centrifugation and pre-enriched for CD4+ T cells by negative selection using immunomagnetic sorting with EasySep Human CD4+ T-cell Isolation kit (Stemcell). Live CD4+CD25highCD127low Tregs were sorted from enriched CD4+ T cells on a BD FACSArta™ Fusion Cell Sorter (see Supporting information Table S2 for all antibodies used). Sorted Tregs had a purity of more than 95% and coexpressed FoxP3 (Supporting information Fig. S9C). Sorted Tregs were incubated overnight and then cocultured at a 2:1 ratio with HUVECs in 24 well plates. After 1 h of coculture, HUVECs were stimulated with 0.5 ng/mL of TNF for 6 h. In a separate set of experiments, the Treg conditioned medium from overnight cultures was used in parallel with the Treg cells. In some experiments, 100 ng/mL LAP (RnD) or 5 μg/mL of neutralizing antibodies to IL-10 (clone JES3-19F1; Biologend) was also added to the Treg conditioned media in order to block TGF-β and IL-10 activity, respectively. The release of CXCL9, CXCL10, CXC3CL1, VCAM-1, and ICAM-1 into the culture medium was analyzed using a procartaplex assay from Luminex on a MAGPIX instrument (ThermoFisher Scientific).

Nanostring and RT-PCR analyses

The Nanostring immunology panel (594 genes) was used for targeted mRNA expression analyses at KIGene—genetic analysis at CMM, Stockholm, Sweden. Raw RNA count data were preprocessed by applying background thresholding and content normalization in NanoString nSolver 4.0.
Flow cytometry

Flow cytometry analyses were performed in accordance with Cosarizza et al. [51]. Mouse and human single-cell suspensions and endothelial cell lines were stained with viability stains (see Supporting information Table S2) to exclude dead cells followed by staining of surface expressed proteins. For intracellular staining, cells were either permeabilized with FIX&PERM® Cell Fixation and Permeabilization Kit (Nordic-MÜbio, for intracellular proteins) or eBioscienceTM Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher, for FoxP3 staining) followed by staining of intracellular markers. Samples were acquired on a Fortessa flow cytometer (BD Biosciences) or Cytoflex (Beckman Coulter) and analyzed using FlowJo software (Tree Star Inc.). See Supporting information Fig. S9A,B for gating strategies and Supporting information Table S2 for antibodies used.

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Conflict of interest: Author BSS is a listed inventor in a non-provisional patent application filed by Johns Hopkins Technology Ventures covering the compound PDDC included in this manuscript. All other authors have no commercial or financial conflict of interest.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Ethics approval statements: The study was performed according to the declaration of Helsinki and approved by the Regional Board of Ethics in Medical Research in west Sweden. The animal studies were approved by the Regional Animal Ethics Committee at the University of Gothenburg.

Patient consent statement: The patients participating in the study received oral and written information about the study, and gave written consent to participate.

Author contributions: Conceptualization: PA and MQJ conceptualized the project; PA, LS, VL, VK, PS, and CT designed and performed experiments and interpreted the data; PA, BSS, and MQJ acquired funding; EBL provided clinical resources; CT and BSS provided chemical resources; PA and MQJ wrote the manuscript. All authors contributed to the final drafting of the manuscript.

References

1 Brun, D., Angell, H. K. and Galon, J., The immune contexture and immunoscore in cancer prognosis and therapeutic efficacy. Nat. Rev. Cancer 2020. 20: 662–680.

2 Carman, C. V. and Martinelli, R., T lymphocyte-endothelial interactions: emerging understanding of trafficking and antigen-specific immunity. Front. Immunol. 2015. 6: 603.

3 Angell, H. K., Brun, D., Barrett, J. C., Herbst, R. and Galon, J., The immunoscore: colon cancer and beyond. Clin. Cancer Res. 2020. 26: 332–339.

4 Guo, L., Wang, C., Qiu, X., Fu, X. and Chang, P., Colorectal cancer immune infiltrates: significance in patient prognosis and immunotherapeutic efficacy. Front. Immunol. 2020. 11: 1052.

5 Tosolini, M., Kirilovsky, A., Miecnik, B., Fredriksen, T., Mauger, S., Bindea, G., Berger, A. et al., Clinical impact of different classes of infiltrating T cytotoxic and helper cells (Th1, Th2, Treg, Th17) in patients with colorectal cancer. Cancer Res. 2011. 71: 1263–1271.

6 Saito, T., Nishikawa, H., Wada, H., Nagano, Y., Sugiyama, D., Atarashi, K., Maeda, Y. et al., Two FOXP3(+)/CD4(+) T cell subpopulations distinctly control the prognosis of colorectal cancers. Nat. Med. 2016. 22: 679–684.

7 Tanaka, A. and Sakaguchi, S., Regulatory T cells in cancer immunotherapy. Cell Res. 2017. 27: 109–118.

8 Buchbinder, E. I. and Desai, A., CTLA-4 and PD-1 Pathways: similarities, differences, and implications of their inhibition. Am. J. Clin. Oncol. 2016. 39: 98–106.

9 Le, D. T., Uram, J. N., Wang, H., Bartlett, B. R., Kemberling, H., Eyring, A. D., Skora, A. D. et al., PD-1 blockade in tumors with mismatch-repair deficiency. N. Engl. J. Med. 2015. 372: 2509–2520.
Overman, M. J., McDermott, R., Leach, J. L., Lonardi, S., Lenz, H. J., Morse, M. A., Dessi, J. et al., Nivolumab in patients with metastatic DNA mismatch repair-deficient or microsatellite instability-high colorectal cancer (CheckMate 142): an open-label, multicentre, phase 2 study. *Lancet Oncol.* 2017. 18: 1182–1191.

Overman, M. J., Lonardi, S., Wong, K. Y. M., Lenz, H. J., Gelsominio, F., Aglietta, M., Morse, M. A. et al., Durable clinical benefit with nivolumab plus ipilimumab in DNA mismatch repair-deficient/microsatellite instability-high metastatic colorectal cancer. *J. Clin. Oncol.* 2018. 36: 773–779.

Onda, M., Kobayashi, K. and Pastan, I., Depletion of regulatory T cells in tumors with an anti-CD25 immunomodin induces CD8 T cell-mediated systemic antitumor immunity. *Proc. Natl. Acad. Sci. U. S. A.* 2019. 116: 4575–4582.

Le Guvello, S., Bastuji-Garin, S., Aloubou, N., Mansour, H., Chaunette, M. T., Berrehaar, F., Seikour, A. et al., High prevalence of Foxp3 and IL17 in MMR-proficient colorectal carcinomas. *Gut* 2008. 57: 772–779.

Michel, S., Benner, A., Tarivderian, M., Wentzensen, N., Hoefler, P., Pommereunck, T., Grabe, M. et al., High density of Foxp3-positive T cells infiltration colorectal cancers with microsatellite instability. *Br. J. Cancer* 2008. 99: 1867–1873.

Svensson, H., Olsson, V., Lundin, S., Yokkala, C., Bjorkc, S., Borjeson, L., Gustavsson, B. et al., Accumulation of CCR4(+)/CTLA4-Foxp3(+)CD25(high) regulatory T cells in colon adenocarcinomas correlate to reduced activation of conventional T cells. *PloS One* 2012. 7: e30695.

Han, S., Toker, A., Liu, Z. Q. and Ohashi, P. S., Turning the tide against regulatory T cells. *Front. Oncol.* 2019. 9: 279.

Akeus, P., Szepońek, L., Ahlmanne, F., Sundstrom, P., Alsen, I., Gustavsson, B., Sparwasser, T. et al., Regulatory T cells control endothelial chemokine production and migration of T cells into intestinal tumors of APC(min/+) mice. *Cancer Immunol. Immunother.* 2018. 67: 1067–1077.

Szepoń, L., Akeus, P., Rodin, W., Raghavan, S. and Quading-Jarbrink, M., Regulatory T cells specifically suppress conventional CD8alphaB T cells in intestinal tumors of APC(min+) mice. *Cancer Immunol. Immunother.* 2020. 69: 1279–1292.

Teng, M. W., Ngiow, S. F., von Scheidt, B., McLaughlin, N., Sparwasser, T. and Smyth, M. J., Conditional regulatory T-cell depletion releases adaptive immunity preventing carcinogenesis and suppressing established tumor growth. *Cancer Res.* 2010. 70: 7800–7809.

Li, X., Kostareli, E., Suffner, J., Garbi, N. and Hammerling, G. J., Efficient Treg depletion induces T-cell infiltration and rejection of large tumors. *Eur. J. Immunol.* 2010. 40: 3325–3335.

Akeus, P., Langenes, V., Kristensen, J. von, Mentzer, A., Sparwasser, T., Raghavan, S. and Quading-Jarbrink, M., Treg-cell depletion promotes chemokine production and accumulation of CXCR3(+) conventional T cells in intestinal tumors. *Eur. J. Immunol.* 2015. 45: 1654–1666.

Sundstrom, P., Stenstad, H., Langenes, V., Ahlmanne, F., Theander, L., Ndah, T. G., Fredin, K. et al., Regulatory T cells from colon cancer patients inhibit effector T-cell migration through an adenosine-dependent mechanism. *Cancer Immunol. Res.* 2016. 4: 183–193.

Maganto-Garcia, E., Bu, D. X., Turro, M. L., Alcaye, P., Newton, G., Griffin, G. K., Croce, K. J. et al., F03p3-inducible regulatory T cells suppress endothelial activation and leukocyte recruitment. *J. Immunol.* 2011. 187: 3521–3529.

Bhati, R., Patterson, C., Livasy, C. A., Fan, C., Ketelsen, D., Hu, Z., Reynolds, E. et al., Molecular characterization of human breast tumor vascular cells. *Am. J. Pathol.* 2008. 172: 1381–1390.

Hertervig, E., Nilsson, A., Nyberg, L. and Duan, R. D., Alkaline sphingomyelinase activity is decreased in human colorectal carcinoma. *Cancer* 1997. 79: 448–453.
CD8+ T cells, natural killer cells, and dendritic cells in breast carcinoma. 

J. Surg. Oncol. 2012. 106: 386–392.

41 Moser, A. R., Luongo, C., Gould, K. A., McNeley, M. K., Shoemaker, A. R. and Dove, W. F., ApcMin: a mouse model for intestinal and mammary tumorigenesis. Eur. J. Cancer 1995. 31A: 1061–1064.

42 Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A. et al., Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J. Exp. Med. 2007. 204: 57–63.

43 Rojas, C., Sala, M., Thomas, A. G., Datta Chaudhuri, A., Yoo, S. W., Li, Z., Dash, R. P. et al., A novel and potent brain penetrant inhibitor of extracellular vesicle release. Br. J. Pharmacol. 2019. 176: 3857–3870.

44 Lee, M., Kiefel, H., LaJevic, M. D., Macauley, M. S., Kawashima, H., O’Hara, E., Pan, J. et al., Transcriptional programs of lymphoid tissue capillary and high endothelium reveal control mechanisms for lymphocyte homing. Nat. Immunol. 2014. 15: 982–995.

45 Sala, M., Hollinger, K. R., Thomas, A. G., Dash, R. P., Tallon, C., Veeravalli, V., Lovell, L. et al., Novel human neutral sphingomyelinase 2 inhibitors as potential therapeutics for Alzheimer disease. J. Med. Chem. 2020.

46 Rodin, W., Sundstrom, P. and Quding Jarbrink, M., Isolation and characterization of MAIT cells from tumor tissues. Methods Mol. Biol. 2020. 2098: 39–53.

47 Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W. and Smyth, G. K., limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic. Acids. Res. 2015. 43: e47.

48 Law, C. W., Alhamdoosh, M., Su, S., Dong, X., Tian, L., Smyth, G. K. and Ritchie, M. E., RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. F1000Res 2016. 5.

49 Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L., Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 2012. 13: 134.

50 Pfaffl, M. W., A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids. Res. 2001. 29: e45.

51 Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W. et al., Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). Eur. J. Immunol. 2019. 49: 1457–1973.

Abbreviations:
CRC: colorectal cancer · DT: diphtheria toxin · DPTIP: 2,6-dimethoxy-4-(5-phenyl-4-thiophen-2-yl-1H-imidazol-2-yl)-phenol · HMVEC: human microvascular endothelial cells · HUVEC: human umbilical vein endothelial cells · IRF1: interferon regulatory factor 1 · LAP: latency-associated peptide · nSMase2: neutral sphingomyelinase 2 · S1P: sphingosine-1-phosphate

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