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CD103 is a hallmark of tumor-infiltrating regulatory T cells

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Regulatory T cells (Treg) mediate tolerance towards self-antigens by suppression of innate and adaptive immunity. In cancer patients, tumor-infiltrating FoxP3+ Treg suppress local anti-tumor immune responses and are often associated with poor prognosis. Markers that are selectively expressed on tumor-infiltrating Treg may serve as targets for immunotherapy of cancer. Here we show that CD103, an integrin mediating lymphocyte retention in epithelial tissues, is expressed at high levels on tumor-infiltrating FoxP3+ Treg in several types of murine cancer. In the CT26 model of colon cancer up to 90% of the intratumoral FoxP3+ cells expressed CD103 compared to less than 20% in lymphoid organs. CD103+ Treg suppressed T effector cell activation more strongly than CD103^{neg} Treg. Expression of CD103 on Treg closely correlated with intratumoral levels of transforming growth factor β (TGF-β) and could be induced in a TGF-β-dependent manner by tumor cell lines. *In vivo*, gene silencing of TGF-β reduced the frequency of CD103+ Treg, demonstrating that CD103 expression on tumor-infiltrating Treg is driven by intratumoral TGF-β. Functional blockade of CD103 using a monoclonal antibody did however not reduce the number of intratumoral Treg, indicating that CD103 is not involved in homing or retention of FoxP3+ cells in the tumor tissue. In conclusion, expression of CD103 is a hallmark of Treg that infiltrate TGF-β-secreting tumors. CD103 thus represents an interesting target for selective depletion of tumor-infiltrating Treg, a strategy that may help to improve anti-cancer therapy.

Regulatory T cells (Treg) are crucial in the prevention of autoimmunity by inhibiting effector T cell responses against self-antigens.[1] Treg however also inhibit immune responses against malignant tumors and thus facilitate cancer development.[2] Indeed, a prominent role of Treg in tumor-associated immunosuppression has been confirmed by several recent studies. During tumor progression Treg accumulate in the blood and lymphoid organs of the tumor-bearing host and in several types of cancer Treg abundantly infiltrate the tumor tissue itself.[2,3] Inhibition of anti-cancer immunity is mediated predominantly by tumor-infiltrating Treg that suppress effector T cell responses locally at the tumor site.[4] The number of tumor-infiltrating FoxP3+ Treg is associated with poor prognosis and has been identified as a significant predictor of patient death in several types of human cancer.[5–7]

Given the detrimental role of Treg in tumor progression, efforts were made to identify target molecules to selectively deplete these cells. The transcription factor FoxP3, the most distinctive marker characterized so far for Treg in both humans and mice, is not accessible to depleting antibodies due to its intracellular expression.[3] Natural thymus-derived Treg constitutively express the interleukin-2 receptor α-chain (CD25) and treatment of mice with monoclonal antibodies against CD25 leads to a temporary reduction of CD4+FoxP3+ cells.[8] This enhances anti-tumor immunity and can lead to T cell dependent rejection of pre-existing tumors.[9] However, with tumor progression the efficacy of anti-CD25 treatment is gradually reduced, a fact that may result from simultaneous depletion of activated CD25-expressing effector T cells.[10] Another antibody-mediated strategy to inhibit Treg function is the activation or blockade of target molecules on these cells without depletion. Activation of the glucocorticoid-induced tumor-necrosis factor receptor related protein (GITR) by an agonistic antibody inhibits Treg function and shows *in vivo* anti-tumor activity.[11,12] A blocking antibody to the cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) expressed by both regulatory and effector T cells inhibits Treg-induced suppression and is currently evaluated in clinical trials.[3,13] Anti-CTLA4 treatment however affects the entire pool of Treg and an important limitation of this approach is the development of systemic autoimmunity.[14] A marker predominantly expressed by

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Abbreviations: CTLA4: cytotoxic T-lymphocyte-associated antigen 4; TGF-β: transforming growth factor β; Treg: regulatory T cell
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tumor-infiltrating Treg would represent a more selective target to enhance anti-cancer immunity.

The plasma membrane-associated molecule CD103, also called $\alpha_E\beta_7$, belongs to the family of integrins and is poorly expressed by immune cells in the spleen or the peripheral lymph nodes.[15] CD103 can be detected mainly on T cell populations within the mucosal epithelium of the gut and on intestinal lamina propria leukocytes.[16] It is thought that CD103 contributes to the retention of lymphocytes in epithelial tissues through interaction with its receptor E-cadherin expressed by epithelial cells.[17] Mice deficient for CD103 have slightly reduced numbers of intestinal intraepithelial lymphocytes, but apart from that are healthy, indicating that CD103 is probably dispensable.[18] Among Treg in lymphoid tissues, a subset of about 20% expresses CD103 and these cells display an effector memory phenotype with low expression levels of CD45RB and high levels of CD44.[19] Some reports further indicate that CD103+ Treg more strongly inhibit CD4 T cell proliferation than conventional Treg.[19,20] Thus, CD103 is a surface-expressed molecule that marks both intestinal lymphocytes and a particularly suppressive subtype of Treg.

In this study, we analyzed tumor-infiltrating Treg for expression of CD103 in four different murine models of cancer. In all tumor models, we found that the majority of intratumoral Treg express CD103 with up to 90% of FoxP3+ Treg staining positive for CD103. High proportions of CD103+ cells were further specific for tumor-infiltrating Treg. CD103 expression on Treg correlated with TGF-β secretion of tumor cells and could be down-regulated by RNAi-mediated gene silencing of TGF-β. Therapeutic targeting of CD103 may represent a promising approach to enhance anti-cancer immunity.

Material and Methods

Mice and cell lines

Female BALB/c and C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). Mice were 5 to 10 weeks of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). The murine cell lines Colon-26 (CT26; Cell Lines Service, Heidelberg, Germany), B16 melanoma F1 (LGC Promochem, Teddington, UK), EL-4 lymphoma (Institute of Immunology, University of Munich) and Panc02 (kindly provided by Prof. C. Bruns, Department of Surgery, University of Munich) were maintained in DMEM medium supplemented with 10% FCS, 1% L-glutamine, 1 U/ml penicillin and 0.1 mg/ml streptomycin (all PAA Laboratories, Coelbe, Germany). For tumor induction 0.25×10^6 (CT26), 1×10^6 (B16 and Panc02), or 5×10^6 (EL4) tumor cells were injected subcutaneously into the flank. The tumor-draining lymph nodes were identified by connecting vessels, the proximity to the tumor and the larger size compared to non-draining lymph nodes. Tumor size was expressed as the product of the perpendicular diameters of individual tumors (mm^2). Tumor growth was calculated as final tumor size divided by the number of days since injection (mm^2/d) to normalize data from independent experiments.

Immunohistology

Tumor tissues were frozen in liquid nitrogen and 5 μm cryosections from the center of the tumors were prepared. The following primary antibodies were used: anti-mouse CD103 (Biolegend, San Diego, CA), anti-mouse FoxP3 (Ebioscience, San Diego, CA) and anti-mouse E-Cadherin (Cell Signaling Technology, Beverly, MA). Cy5 F(ab)2 goat anti-Armenian hamster IgG, biotin F(ab)2 donkey anti-rat IgG, biotin IgG donkey anti-rabbit IgG and rhodamin red X streptavidin were used as detection reagents. Nucleic counterstaining was performed using DAPI (Sigma Aldrich, Steinheim, Germany). Counting was performed blinded by two independent investigators. Images were obtained by fluorescence microscopy (Axiovert 2000 Carl Zeiss, Jena, Germany; 40-fold magnification) using Carl Zeiss Axiovision software and processed with Adobe Photoshop for adjustment of contrast and size.

Flow cytometry

For flow cytometry analysis single cell suspensions of spleen, lymph nodes or Peyer's patches were prepared. Bone marrow cells were harvested from murine femur and tibia and erythrocytes were lysed with ammonium chloride buffer (BD Biosciences). To isolate lymphocytes from tumor, lung, liver or heart, the tissues were mechanically disrupted, incubated with 1 mg/ml collagenase and 0.05 mg/ml DNAse (both Sigma Aldrich) and subsequently passed through a cell strainer. Single cell suspensions were resuspended in 44% Percoll (Biochrome, Berlin, Germany) and layered over 67% Percoll prior to centrifugation at 800 g for 30 min. Lymphocytes from the interphase were stained for flow cytometry. The following antibodies were used: Pacific Blue or PerCP anti-mouse CD3, PE anti-mouse B220, PE-Cy7 or PerCP anti-mouse CD4, APC-Cy7 anti-mouse CD8 (all Biolegend), FITC anti-mouse CD103 (BD Biosciences, Heidelberg, Germany) and Pacific Blue or APC anti-mouse FoxP3 (Ebioscience). Intracellular detection of FoxP3 was performed using premixed regulatory T cell staining reagents (Ebioscience). Events were measured on a FACS Calibur or FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR).

FACS sorting and proliferation assays

Untouched CD4+ T cells were sorted from single cell suspensions of lymph nodes by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with labeled antibodies against CD103 (Fitc, BD Biosciences), CD4 (PerCP) and CD25 (APC, both from Biolegend). CD4+ CD25+ CD103+, CD4+CD25+CD103^{neg} and CD4+ CD25^{neg} cells were obtained by FACS sorting using a FACS Aria cell sorter (BD Biosciences) with a purity of more than 99%. Treg subsets (3×10^4 cells) and T effector cells (7.5×10^4 cells) were cultured in triplicate with anti-CD3-CD28 beads (Invitrogen, Carlsbad, CA) at a bead-to-cell ratio of 1:5

for 72 hr and in the presence of BrdU (Roche Diagnostics, Mannheim, Germany). To detect proliferation of T effector cells, co-cultures were then stained with Pacific Blue-labeled FoxP3 antibody, fixed with 1% PFA in PBS, incubated with DNAse I (0.05 mg/ml in PBS; Sigma-Aldrich, Steinheim, Germany) and further stained with anti-BrdU-FITC antibody (Invitrogen). T effector cell proliferation and activation was determined by BrdU and CD69 expression of CD4+FoxP3^{neg} cells. IL-2 levels in the co-culture were measured by ELISA (BD Biosciences).

TGF-β in vitro assays

To assess CD103 induction on Treg, 1.2×10^5 splenocytes were cultured in triplicate with supernatants of CT26 or EL-4 tumor cells in the presence of anti-CD3-CD28 coated microbeads (Invitrogen, bead to cell ratio 1:10). Recombinant TGF-β1 or anti-TGF-β1 antibody (both R&D Systems, Minneapolis, MN) were added in a concentration of 5 ng/ml and 12.5 μg/ml, respectively. Cells were cultured for three days before analysis by flow cytometry.

TGF-β ELISA of supernatants and tissue lysates

To measure TGF-β secretion by different tumor cell lines, 5×10^6 tumor cells were plated in 3 ml of medium, cultured for two days and supernatants were analyzed by ELISA (R&D Systems). For analysis of tissues, tumor or lymph node homogenates were resuspended in lysis buffer (BioRad Laboratories, Hercules, CA) and centrifuged. Total protein concentration was measured by Bradford assay (BioRad Laboratories). All samples were diluted to equal protein concentrations and TGF-β1 levels were measured by ELISA. The final cytokine concentration was calculated as ng cytokine/g protein in the respective lysate.

TGF-β gene silencing and in vivo CD103 blocking

For *in vivo* gene silencing of TGF-β, siRNAs were designed according to published guidelines.[21] 3′-Overhangs were carried out as two deoxythymidine residues (dTdT). RNAs were all from Eurofins MWG Operon (Penzberg, Germany). Sequences were: Control RNA: 5′-GAUGAACUUCAGGGUCAGCG-3′ (sense), 5′-CGCUGACCCUGAAGUUCAUC-3′ (antisense); TGF-β1 siRNA: 5′-GAACUCUACCAGAAAUAUAUU-3′ (sense), 5′-AAUAUAUUUCUGGUAGAGUUC-3′ (antisense). Nonsilencing siRNA (control RNA) was designed to contain random sequences that do not match within the murine or human genome. For *in vivo* delivery 50 μg of siRNA was complexed with *in Vivo* JetPEI reagent (Peqlab, Erlangen, Germany) according to the manufacturer's instructions and injected into the tail vein. For *in vivo* blocking of CD103, 150 μg of rat IgG2aκ anti-mouse CD103 antibody (clone M290, Bioxcell, West Lebanon, NH) was injected intraperitoneally.

Statistics

All data are presented as mean +/− SEM and were analyzed as appropriate by unpaired Student's *t*-test or by ANOVA test using the Student Newman Keuls test. Statistical analysis was performed using SPSS software.

Results

A high proportion of tumor-infiltrating FoxP3+ regulatory T cells expresses CD103

To investigate CD103 expression levels on tumor-infiltrating Treg, subcutaneously induced murine CT26 colon and B16 melanoma tumors were examined by immunofluorescence staining. Tissue sections were double-stained with antibodies directed against FoxP3 and CD103 and the proportion of CD103+ Treg was evaluated by counting non-overlapping visual fields from tumors of eleven different mice (Fig. 1*a*). Strikingly, in CT26 tumors more than 90% of the tumor-infiltrating FoxP3+ cells expressed the integrin CD103 (Fig. 1*b*). In contrast, in the spleen only 20% of FoxP3+ cells were positive for CD103, consistent with previous reports.[22] In subcutaneous B16 melanoma tumors, more than 50% of intratumoral Treg expressed CD103 (Fig. 1*b*). We further used flow cytometry to analyze CD103 levels on tumor-infiltrating FoxP3+ cells and could confirm the high proportion of CD103+ Treg in both CT26 and B16 tumors (Fig. 1*c*). Analysis of an additional tumor model, the subcutaneous EL-4 lymphoma, revealed a lower percentage of CD103+ cells within tumor-infiltrating Treg (31%) compared to the CT26 and B16 tumors. In all analyzed models, however, CD103 expression of intratumoral Treg was significantly higher compared to FoxP3+ cells in the spleen of the same mice (Fig. 1*c*). Further, expression levels of CD103 by tumor-infiltrating Treg clearly correlated with tumor growth (Fig. 1*d*). To confirm previous reports indicating that CD103+ cells represent a particularly suppressive subset of Treg[19,20] we isolated CD103+ and CD103^{neg} Treg from CT26 tumor-bearing mice. Indeed, CD103+ Treg more strongly suppressed proliferation, activation and IL-2 release of T effector cells (Fig. 1*e*). In conclusion, tumor-infiltrating Treg are characterized by high expression levels of CD103, a marker predicting potent suppressive function of these cells.

High expression of CD103 is specific for tumor-infiltrating regulatory T cells

To assess whether the high proportions of CD103+ cells are specific for tumor-infiltrating Treg, we determined the percentage of CD103+ cells within FoxP3+ cells derived from different organs of tumor-bearing mice. In the peripheral lymph nodes, numbers of CD103+ cells among Treg were generally low with a proportion of less than 25% (Fig. 2*a*). Interestingly, in CT26 tumor-bearing mice a significantly higher number of CD103-expressing Treg was detected in the tumor-draining lymph nodes. To assess CD103 expression in

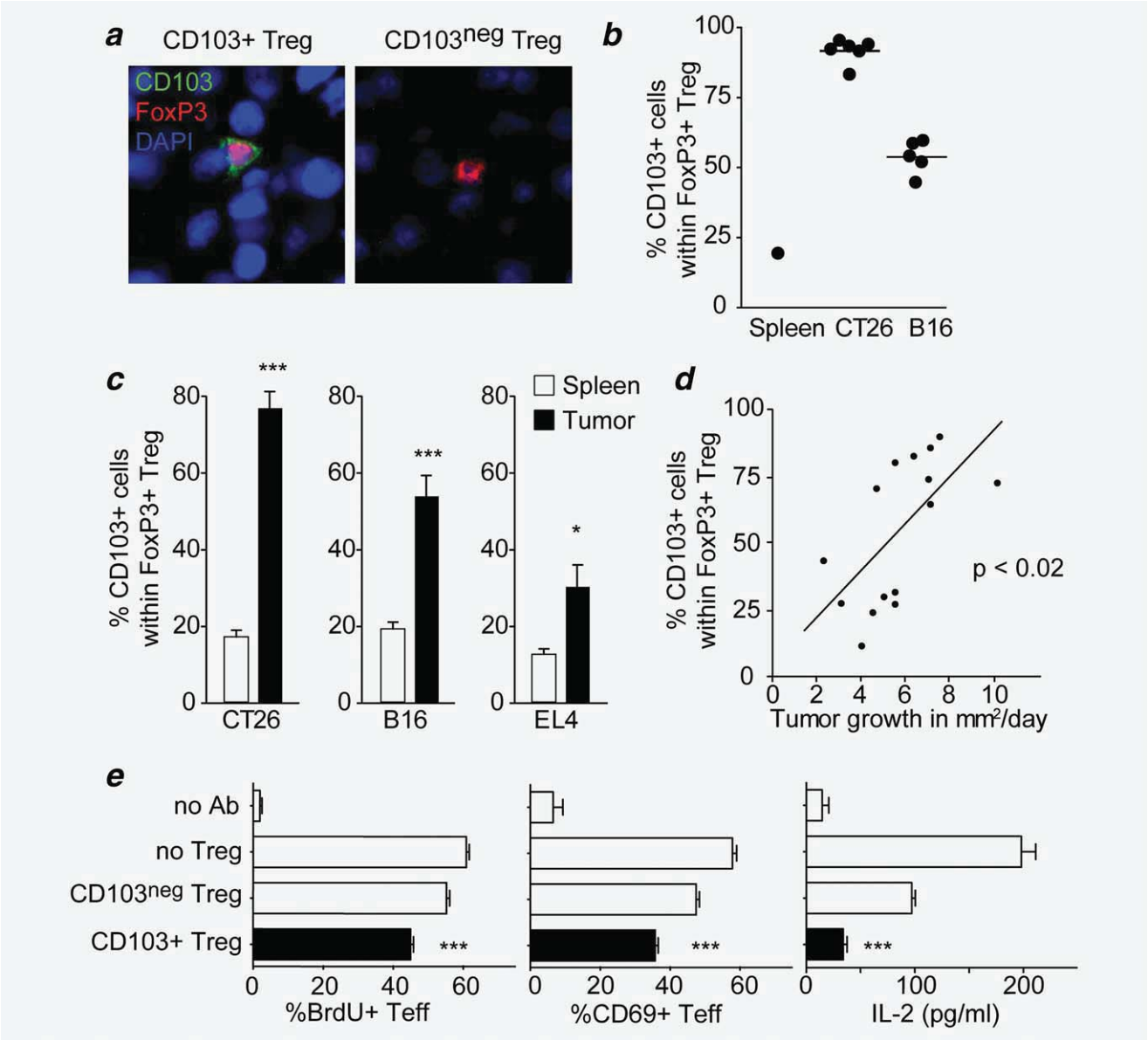

Figure 1. CD103 is highly expressed by tumor-infiltrating Treg. CT26, B16, and EL-4 tumors were induced by subcutaneous injection of the respective tumor cell line. Tumors were isolated and analyzed by immunohistology or flow cytometry when they reached a mean size of 100 mm^2. (*a*) Tissue sections of CT26 tumors were stained for CD103 (green) and FoxP3 (red) and representative images of CD103+ and $CD103^{neg}$ Treg are shown. (*b*) The proportion of CD103+ cells within FoxP3+ cells was determined in CT26 ($n = 6$) and B16 tumors ($n = 5$) and in the spleen of a C57BL/6 mouse ($n = 1$). Each data point represents the proportion of CD103+ cells within FoxP3+ cells in the tumor or the spleen of one mouse. (*c*) Expression of CD103 was determined by flow cytometry on CD4+FoxP3+ cells isolated from the spleen and the tumor tissue of CT26 ($n = 6$), B16 ($n = 5$), or EL-4 ($n = 6$) tumor-bearing mice. The mean percentage of CD103+ cells among CD4+FoxP3+ cells for all mice is shown. (*d*) Tumor growth of CT26 tumors in mm^2/day from three independent experiments was correlated to CD103 expression of CD4+FoxP3+ tumor-infiltrating cells according to Pearson's test. (*e*) CD4+CD25+CD103+ and $CD4+CD25+CD103^{neg}$ Treg as well as $CD4+CD25^{neg}$ T effector cells were isolated by FACS sorting from the tumor-draining lymph nodes of CT26 tumor-bearing mice. T effector cells were co-cultured with either Treg subset in the presence of anti-CD3-CD28 antibody. Proliferation and activation of T effector cells was measured by incorporation of BrdU, expression of CD69 and secretion of IL-2 using flow cytometry or ELISA. Error bars indicate SEM. *P* values for (*b* and *c*) were calculated relative to the proportion of CD103+ cells in the spleen and for (*e*) relative to suppression by $CD103^{neg}$ Treg (*$p < 0.05$; ***$p < 0.001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

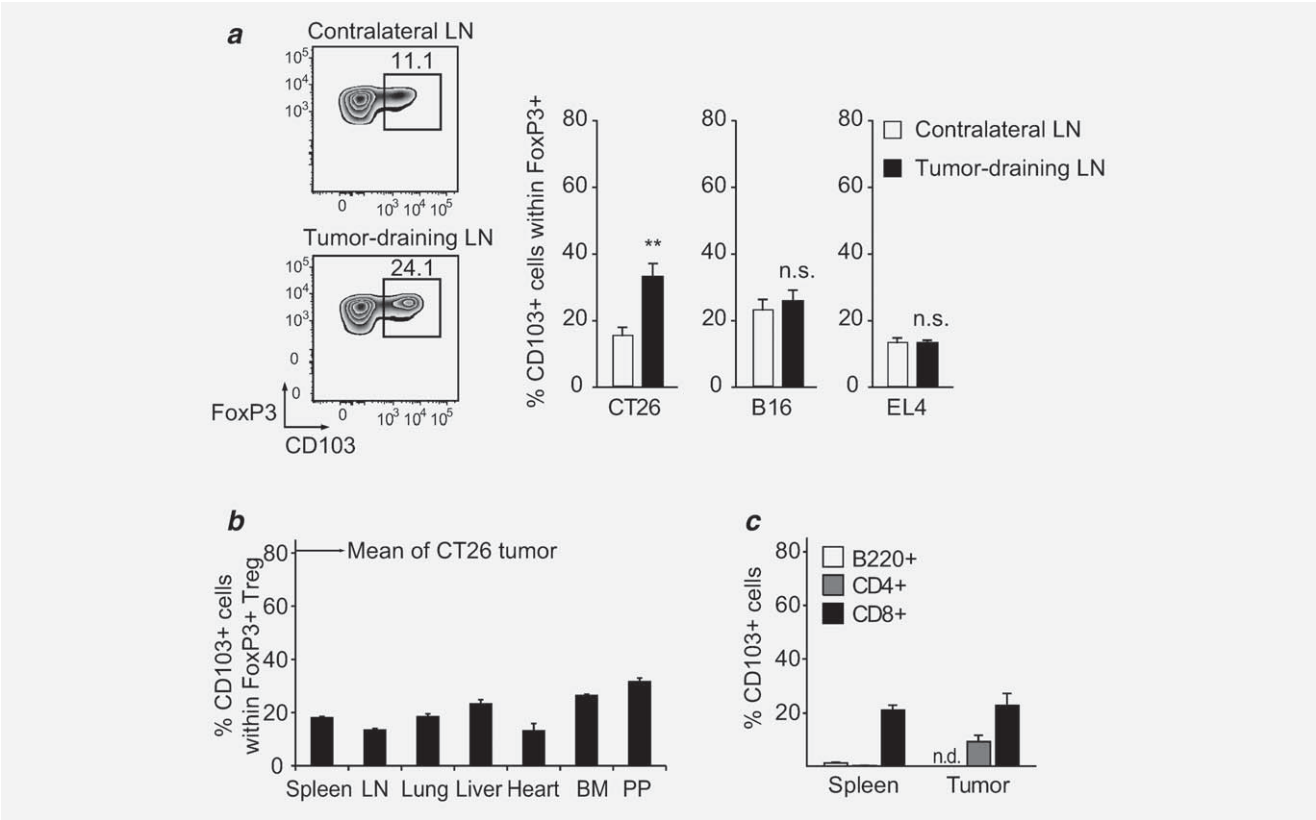

Figure 2. High expression of CD103 is characteristic for tumor-infiltrating Treg. (*a*) Subcutaneous tumors were induced as described in Figure 1 and lymph nodes were isolated when tumors reached a mean size of 100 mm^2. Expression of CD103 on CD4+FoxP3+ cells derived from both tumor-draining and contralateral lymph nodes was analyzed by flow cytometry (CT26, $n = 6$; B16, $n = 5$ and EL-4, $n = 6$). *p* values were calculated relative to the proportion of CD103+ cells in the contralateral lymph node (**$p < 0.01$). (*b*) The bar diagram shows the proportion of CD103+ cells among CD4+FoxP3+ cells isolated from the indicated organs of healthy BALB/c mice ($n = 6$; LN, lymph nodes; BM, bone marrow and PP, Peyer's patches). (*c*) The proportion of CD103+ cells among B220+, CD4+FoxP3^{neg} and CD8+ cells derived form the spleen and the tumor of CT26-bearing mice was analyzed by flow cytometry. Error bars indicate SEM.

other compartments, we isolated lymphocytes from lung, heart, liver, Peyer's patches, and bone marrow and determined CD103 expression by Treg. In all organs analyzed, the proportion of CD103+ cells among Treg was comparable to those in the peripheral lymph nodes (Fig. 2*b*). To further determine the proportion of CD103+ cells among other lymphocyte subsets, we analyzed B220+, CD4+FoxP3^{neg}, and CD8+ cells. Expression of CD103 was observed only on a small proportion of all lymphocyte subtypes, both in the spleen and the tumor tissue (Fig. 2*c*). Thus, high numbers of CD103+ cells are specific for tumor-infiltrating Treg.

Expression of CD103 in tumor-infiltrating regulatory T cells is driven by intratumoral transforming growth factor β

We next examined the mechanism responsible for enhanced CD103 expression on tumor-infiltrating Treg. In gut-associated lymphoid tissue, CD103 expression by lymphocytes is known to be induced by transforming growth factor β (TGF-β)[16] and thus, we hypothesized that cancer-associated TGF-β may give rise to high CD103 levels on tumor-infiltrating Treg. As significant differences were seen in the proportion of CD103+ cells among Treg within different tumor models (Fig. 1*c*), we quantified tumor-associated TGF-β levels to establish a possible relation. Cell culture supernatants as well as tissue lysates of CT26, B16, and EL-4 tumors were analyzed by ELISA. Whereas high and intermediate levels of TGF-β were detected in the supernatants of CT26 and B16 cells, respectively, EL-4 tumor cells did not produce this growth factor (Fig. 3*a*). *In vivo*, a similar pattern of TGF-β levels was observed, with the lowest levels of this cytokine in EL-4 tumors (Fig. 3*b*). Both *in vitro* and *in vivo* levels of TGF-β correlated with CD103 expression by tumor-infiltrating Treg, with high levels of both parameters in CT26 and B16 tumors. As CD103+ Treg were more frequent in tumor-draining than in contralateral lymph nodes we also compared TGF-β levels in these organs. Indeed, in the tumor-draining lymph nodes we detected significantly higher amounts of TGF-β than in non-draining lymph nodes in all but one mouse, thus confirming the positive correlation with CD103 expression (Fig. 3*c*).

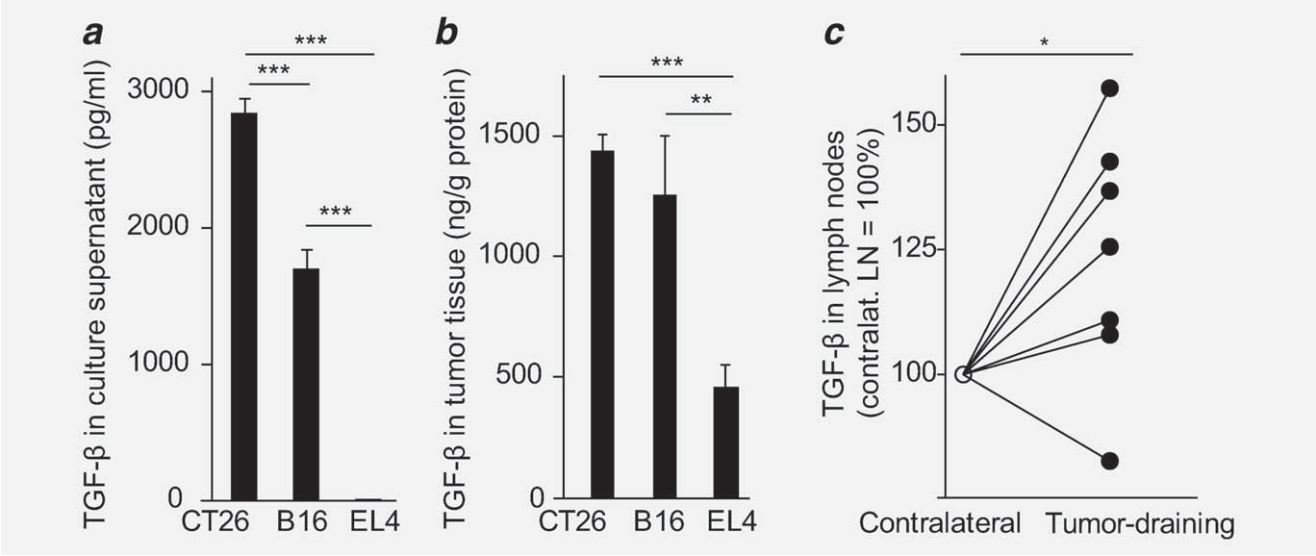

Figure 3. Tumor-derived TGF-β secretion correlates with CD103 expression by Treg. (*a*) Equal numbers of CT26, B16 and EL-4 tumor cells were cultured in triplicate for two days and TGF-β levels in the supernatant were determined by ELISA. (*b* and *c*) Subcutaneous tumors were induced as described in Figure 1 and isolated when they reached a mean size of 100 mm^2. TGF-β levels were determined by ELISA in tissue lysates of the respective tumors (CT26, $n = 6$; B16, $n = 4$ and EL-4, $n = 5$) and of the tumor-draining and contralateral lymph nodes derived from CT26-bearing mice. The concentration of TGF-β in the tumor-draining lymph nodes is shown relative to the contralateral lymph nodes (set at 100%) for each mouse. Error bars indicate SEM. *p* values were calculated as indicated by the lines (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

To investigate whether tumor-associated TGF-β triggers CD103expression by Treg, we cultured splenocytes in supernatants from CT26 tumor cells and quantified the proportion of CD103+ cells within CD4+FoxP3+ Treg. Indeed, CT26 supernatants induced CD103 expression by Treg and blocking of TGF-β efficiently prevented CD103 induction (Fig. 4*a*). In contrast to CT26, supernatants of TGF-β-negative EL-4 tumor cells did not induce CD103 on Treg. A clear up-regulation of CD103 on Treg was however observed upon addition of recombinant TGF-β to EL-4 supernatants (Fig. 4*b*) or by recombinant TGF-β alone (not shown). These data demonstrate that CD103 expression on Treg is directly triggered by tumor cell-derived TGF-β. Interestingly, this effect was specific for FoxP3+ Treg, as no induction of CD103 was seen on CD4+$FoxP3^{neg}$ or CD8+ T cells (Fig. 4*c*). The role of TGF-β in CD103 induction on Treg was further assessed *in vivo* by siRNA-mediated knock-down. Treatment of tumor-bearing mice with a TGF-β siRNA significantly reduced intratumoral levels of TGF-β, as determined by ELISA of tissue lysates (Fig. 4*d*). We then analyzed CD103 expression by tumor-infiltrating FoxP3+ cells using immunohistology. In mice treated with a control RNA nearly 50% of intratumoral Treg expressed CD103. Strikingly, a significant decrease of CD103 expression by Treg was observed upon treatment with the TGF-β-specific siRNA (Figs. 4*e* and 4*f*). In conclusion, expression of CD103 by tumor-associated Treg is driven by TGF-β.

CD103 is not required for the retention of regulatory T cells in the tumor

CD103 is an integrin that mediates retention of lymphocytes in epithelial tissues.[17] To assess whether expression of CD103 is necessary for the homing and retention of Treg in malignant tumors, we treated tumor-bearing mice with a blocking antibody against CD103. The monoclonal rat anti-mouse CD103 antibody (clone M290) binds to the α_E-subunit and blocks the interaction of CD103 with its receptor E-cadherin, but does not deplete CD103+ cells.[23,24] Treatment with anti-CD103 antibody was started one week after tumor induction and infiltration by FoxP3+ cells was evaluated 10 days later. We observed no differences in the number of tumor-infiltrating FoxP3+ cells between untreated and anti-CD103 treated mice (Fig. 5*a*). Efficient delivery of the antibody was confirmed by showing *in vivo* binding of M290 to CD103; this was demonstrated by staining frozen tumor sections of treated mice with a fluorescence-labeled anti-rat antibody (Fig. 5*b*). Anti-CD103 treatment further did not alter Treg or CD8 T cell numbers in the tumor-draining lymph nodes (Figs. 5*c* and 5*d*) or the spleen (data not shown) and had no impact on tumor growth (Fig. 5*e*). In addition, we found that the only known receptor for CD103, E-cadherin, is not expressed within CT26 tumors (Fig. 5*f*). Thus, although the majority of FoxP3+ cells expresses CD103, this integrin appears not to be required for the retention of Treg in the tumor tissue. Therapeutic targeting of CD103+ cells will therefore require the use of an antibody with depleting rather

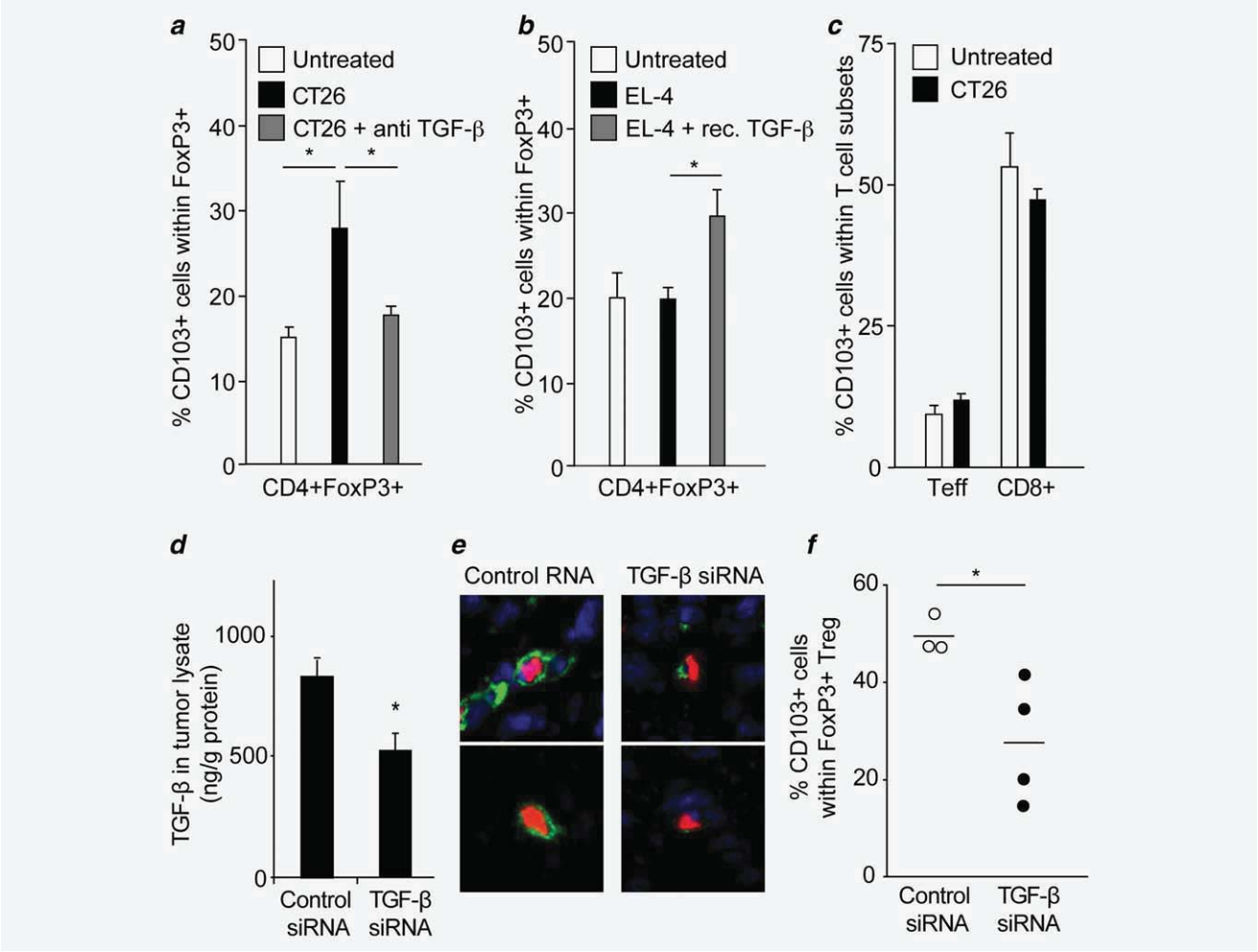

Figure 4. Expression of CD103 on tumor-infiltrating Treg is mediated by TGF-β. (*a–c*) Freshly isolated splenocytes (1.2×10^5) were stimulated with anti-CD3-CD28 coated microbeads and cultured with supernatants of CT26 or EL-4 tumor cells in the presence of either TGF-β blocking antibody or recombinant TGF-β. CD103 expression on CD4+FoxP3+, CD4+FoxP3neg (Teff) and CD8+ T cells was determined by flow cytometry after three days. (*d–f*) Subcutaneous Panc02 tumors of C57BL/6 mice were treated with a siRNA (50 μg i.v.) directed against TGF-β1 ($n = 4$) or with an irrelevant control RNA ($n = 3$) 10 days after induction. siRNA treatment was repeated after 36 hr and 12 hr later tumors were removed for analysis by ELISA and immunohistology. TGF-β levels were determined by ELISA in tissue lysates of RNA-treated tumors (*d*). Tumor sections were double-stained for CD103 (green) and FoxP3 (red) and two representative images are shown for both control RNA and TGF-β siRNA-treated mice (*e*). The proportion of CD103+ cells within FoxP3+ cells was determined in both groups by counting non-overlapping visual fields. Each data point represents the mean proportion of CD103+ cells within FoxP3+ Treg in the tumor of one mouse and bars indicate the mean of one treatment group (*f*). Error bars indicate SEM. *p* values were calculated relative to control RNA-treated mice (*$p < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

than blocking function. The development of such an antibody could be a promising approach for cancer immunotherapy.

Discussion

Treg inhibit immune responses against malignant tumors and represent an important obstacle for cancer immunotherapy.[3] In particular, Treg infiltrating the tumor tissue itself inhibit anti-cancer immunity and correlate with poor prognosis in many types of human cancer.[4,5,25] A comprehensive knowledge of the phenotype of tumor-infiltrating Treg is crucial to understand their mode of action and to develop therapeutic strategies that target these cells. In this study, we demonstrate that the majority of tumor-infiltrating Treg expresses CD103, a cell surface protein of the integrin family. In CT26 tumors more than 90% of Treg expressed CD103 and a high proportion of CD103+ cells within intratumoral FoxP3+ Treg was observed in three other models of murine cancer. In contrast, analysis of CD103 expression by Treg in a broad panel of peripheral organs including the spleen, lymph nodes, lung, liver, heart, bone marrow, and Peyer's patches revealed

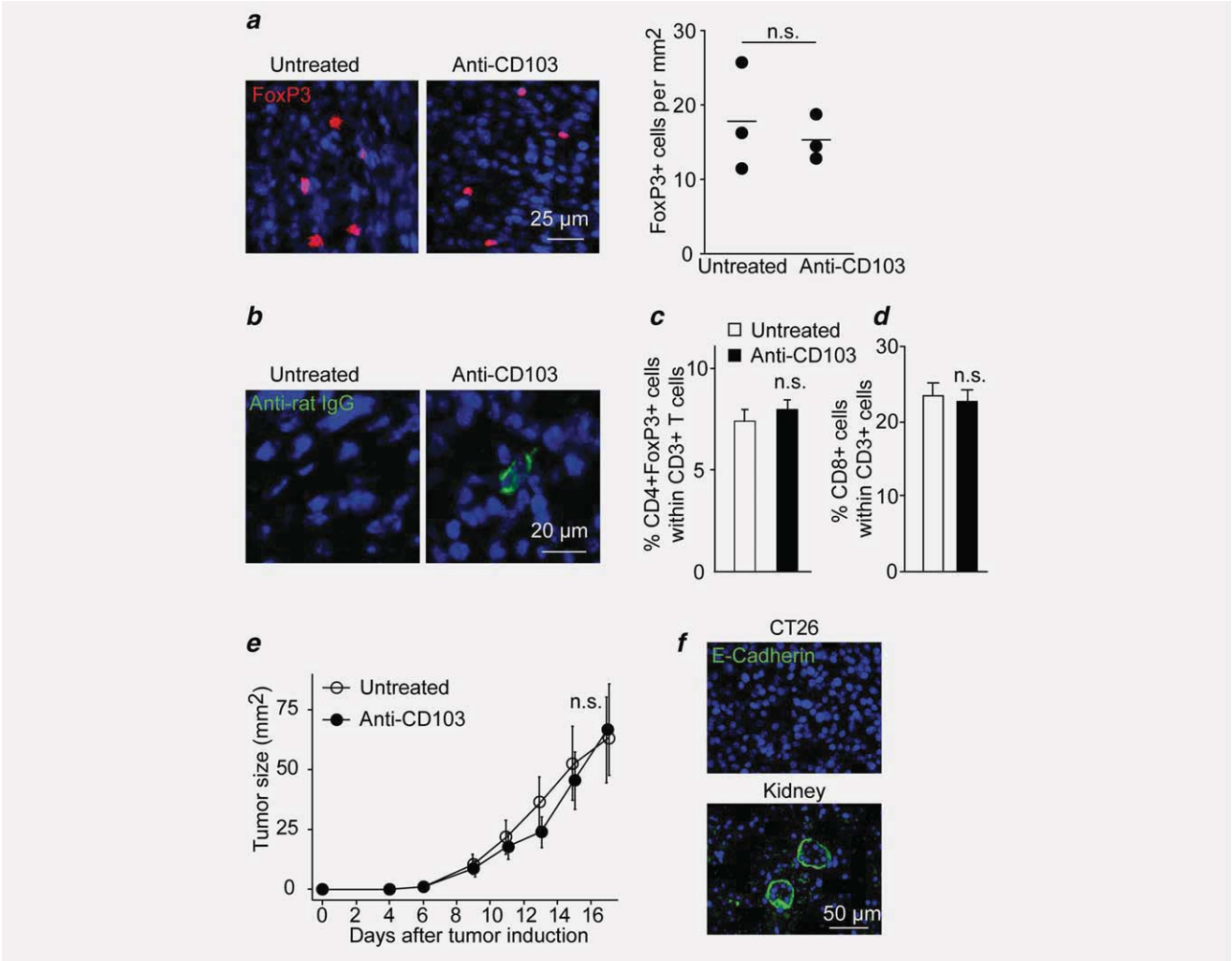

Figure 5. CD103 is not required for the retention of Treg in the tumor tissue. (*a–e*) Seven days after induction of subcutaneous CT26 tumors mice received intraperitoneal injections of 150 µg monoclonal rat anti-mouse CD103 antibody every second day (clone M290) or remained untreated ($n = 6$ for each group). Tumors were removed ten days after the first treatment and analyzed by immunohistology. (*a*) Representative images and quantification of tumor-infiltrating FoxP3+ cells ($n = 3$ for each group). (*b*) Frozen tissue sections of untreated and anti-CD103-treated tumors were stained with biotin-conjugated anti-rat IgG antibody, followed by fluorescence-conjugated streptavidin (green) for the *in vivo* detection of the monoclonal rat-anti mouse CD103 antibody. (*c* and *d*) The proportion of CD4+FoxP3+ cells and CD8+ cells within CD3+ cells in the tumor-draining lymph node was determined by flow cytometry. (*e*) Tumor growth of both untreated and anti-CD103 treated mice was determined. Error bars indicate SEM. *p* values were calculated relative to untreated mice (n.s., not significant). (*f*) Untreated tumor tissues of CT26 tumors (experiment described in Fig. 1) were analyzed by immunohistology for the expression of E-cadherin (green) and kidney sections from healthy mice were used as positive control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

only low expression levels with about 20% CD103+ cells among FoxP3+ Treg. Thus, in cancer-bearing hosts high expression of CD103 is a unique property of tumor-infiltrating Treg. Previous work has shown that positivity for CD103 defines a subset of Treg with specific migratory and anti-inflammatory properties: CD103+ Treg express a set of chemokine receptors similar to activated T cells and thus preferentially home to sites of inflammation.[22] The specific pattern of chemokine receptor expression could thus explain the accumulation of these cells in malignant tumors. An important property of CD103+ Treg is their highly suppressive function: CD25+CD103+ Treg inhibit effector T cell proliferation more potently than CD25+CD103^{neg} cells[19,20] and tumor-derived CD103+ Treg suppress CD8 T cell responses more strongly than CD103^{neg} Treg.[26] The prevalence of CD103+ Treg that we observed in several types of tumors may therefore enhance immunosuppression. We conclude that CD103 represents an interesting molecule to

therapeutically target a tumor-resident and highly immunosuppressive Treg subset.

Due to the central role of Treg in tumor-associated immunosuppression, targeting these cells by specific antibodies is a promising approach. Depleting antibodies against CD25 have been extensively tested and show some efficacy in mice, but lack significant benefits in patients so far.[27] A blocking antibody against CTLA-4, a receptor expressed by T cells, synergistically enhances anti-tumor immunity by inhibiting Treg-induced suppression and promoting T effector cell activation[28]; this treatment can however lead to systemic autoimmunity as CTLA-4 is ubiquitously expressed.[14] For CD103, we have shown predominant expression by Treg in the tumor tissue and to some extent in the tumor-draining lymph node. Thus, targeting this molecule could eliminate Treg at the sites where they most potently suppress tumor-specific immunity. Unfortunately, a depleting antibody against CD103 has so far not been developed. In mice, only antibodies of the rat IgG2b and to some extent of the IgG1 isotype bear the potential to induce antibody-mediated cytotoxicity leading to depletion of target cells.[29,30] In this study we used the monoclonal rat IgG2aκ antibody M290, which blocks the interaction of CD103 with its receptor E-cadherin.[23,24] As expected, M290 treatment did not deplete CD103+ cells. However, we speculated that anti-CD103 treatment could reduce Treg numbers *via* interfering with the retention of Treg within the tumor. The principal function of CD103 is to mediate adhesion of cells in epithelial tissues[17] and for Treg, it has been shown that CD103 is essential for the retention in inflamed skin during infection with the parasite Leishmania major.[31] As Treg numbers were not altered by anti-CD103 treatment, our data show that CD103 is not involved in the retention of Treg within malignant tumors. This is further supported by the lack of E-cadherin expression in the tumor tissue, the so far only identified ligand for CD103. Thus, blocking CD103 is not sufficient to suppress tumor infiltration by Treg, but generation of depleting antibodies will be an interesting approach for immunotherapy of cancer.

As CD103 does not mediate retention of Treg in the tumor tissue, another mechanism must be responsible for the accumulation of intratumoral FoxP3+ cells expressing this integrin. Our data suggest that intratumoral TGF-β promotes the expression of CD103 on tumor-infiltrating Treg. We found that high levels of CD103 were expressed predominantly in those types of tumors with strong TGF-β secretion. In addition, *in vivo* gene silencing of TGF-β reduced the number of intratumoral CD103+ Treg. Our hypothesis is supported by previous reports showing that TGF-β is a potent inducer of CD103 *in vitro*.[32,33] Further, *in vivo* TGF-β mediates expression of CD103 by intraepithelial and lamina propria-associated lymphocytes[16] and induces the generation of CD103-expressing FoxP3+ Treg from naïve T cells.[34] The high number of tumor-infiltrating CD103+ Treg could thus result from the conversion of previously nonregulatory T cells. In conclusion, CD103 represents a good marker to selectively target Treg in TGF-β-secreting tumors and the development of novel depleting antibodies against CD103 may be a promising approach to improve anti-cancer therapy.

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