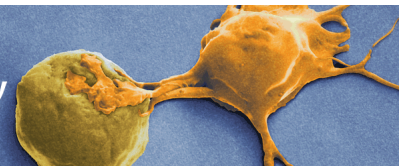




NK Cell Isolation Kits –
best performance from the best in the industry
▶ www.miltenyibiotec.com/nkcells



Angiopoietin 2 Stimulates TIE2-Expressing Monocytes To Suppress T Cell Activation and To Promote Regulatory T Cell Expansion

This information is current as of June 12, 2012.

Seth B. Coffelt, Yung-Yi Chen, Munitta Muthana, Abigail F. Welford, Andrea O. Tal, Alexander Scholz, Karl H. Plate, Yvonne Reiss, Craig Murdoch, Michele De Palma and Claire E. Lewis

J Immunol 2011; 186:4183-4190; Prepublished online 2 March 2011;
doi: 10.4049/jimmunol.1002802
<http://www.jimmunol.org/content/186/7/4183>

-
- Supplementary Material** <http://www.jimmunol.org/content/suppl/2011/03/02/jimmunol.1002802.DC1.html>
- References** This article **cites 31 articles**, 21 of which you can access for free at:
<http://www.jimmunol.org/content/186/7/4183.full#ref-list-1>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at:
<http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/cgi/alerts/etoc>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright © 2011 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Angiopoietin 2 Stimulates TIE2-Expressing Monocytes To Suppress T Cell Activation and To Promote Regulatory T Cell Expansion

Seth B. Coffelt,* Yung-Yi Chen,* Munitta Muthana,* Abigail F. Welford,* Andrea O. Tal,[†] Alexander Scholz,[†] Karl H. Plate,[†] Yvonne Reiss,[†] Craig Murdoch,[‡] Michele De Palma,[§] and Claire E. Lewis*

Angiopoietin 2 (ANGPT2) is a proangiogenic cytokine whose expression is often upregulated by endothelial cells in tumors. Expression of its receptor, TIE2, defines a highly proangiogenic subpopulation of myeloid cells in circulation and tumors called TIE2-expressing monocytes/macrophages (TEMs). Genetic depletion of TEMs markedly reduces tumor angiogenesis in various tumor models, emphasizing their essential role in driving tumor progression. Previously, we demonstrated that ANGPT2 augments the expression of various proangiogenic genes, the potent immunosuppressive cytokine, *IL-10*, and a chemokine for regulatory T cells (Tregs), *CCL17* by TEMs in vitro. We now show that TEMs also express higher levels of *IL-10* than TIE2⁻ macrophages in tumors and that ANGPT2-stimulated release of *IL-10* by TEMs suppresses T cell proliferation, increases the ratio of CD4⁺ T cells to CD8⁺ T cells, and promotes the expansion of CD4⁺CD25^{high}FOXP3⁺ Tregs. Furthermore, syngeneic murine tumors expressing high levels of ANGPT2 contained not only high numbers of TEMs but also increased numbers of Tregs, whereas genetic depletion of tumor TEMs resulted in a marked reduction in the frequency of Tregs in tumors. Taken together, our data suggest that ANGPT2-stimulated TEMs represent a novel, potent immunosuppressive force in tumors. *The Journal of Immunology*, 2011, 186: 4183–4190.

TIE2-expressing monocytes/macrophages (TEMs) are a subpopulation of myeloid cells characterized by the expression of monocyte/macrophage markers as well as the angiopoietin receptor TIE2 (1, 2). TEMs are found in circulation and tumors of both human and mice, and conditional TEM deletion in various mouse tumor models has established their profound effect on tumor angiogenesis and progression (2–4). We recently reported that human circulating TEMs are preprogrammed in the bone marrow or peripheral blood for proangiogenic functions before these cells reach the tumor microenvironment (5). We also found that exposure to angiopoietin 2 (ANGPT2)—a cytokine overexpressed by endothelial cells in tumors (6–8)—further augments the ability of TEMs to stimulate angiogenesis through upregula-

tion of such proangiogenic enzymes as thymidine phosphorylase and cathepsin B (5).

In addition, we demonstrated that ANGPT2 skews the phenotype of human TEMs toward an M2-like macrophage polarization in vitro via downregulation of *IL-6* and increased expression of mannose receptor (*MRC1*), *CCL17*, and *IL-10* (5, 9, 10). TEMs display several characteristics of M2-polarized macrophages in murine tumors (11), and our data indicate that ANGPT2 may drive this phenotype (5). Because M2 macrophages are thought to promote tumor progression through angiogenesis as well as suppression of antitumor immune responses (reviewed in Refs. 10 and 12), we hypothesized that ANGPT2 may stimulate the immunosuppressive properties of TEMs.

In this paper, we show that ANGPT2-stimulated human TEMs suppress T cell proliferation and expand CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Tregs) via *IL-10*-dependent mechanisms. We also demonstrate that murine tumor TEMs express high levels of *IL-10* and are markedly immunosuppressive. These data illustrate a novel protumorigenic role for TEMs.

Materials and Methods

Human leukocyte isolation, coculture, and flow cytometry

Monocytes were isolated from human Buffy coats as described previously (3, 5). Autologous CD3⁺ T cells were isolated by negative selection using StemCell Technologies' Rosettesep kit. CD3⁺ cells were labeled with 10 μ M CFSE (Invitrogen). A 1:1 ratio of 5×10^5 monocytes to 5×10^5 CD3⁺ cells were cocultured in the presence of CD3/28 Dynabeads (Invitrogen) and cytokines for 5 d. Cocultures were also performed with 0.4- μ m Transwell inserts. Recombinant human cytokines (R&D Systems) were added at the following concentrations: 300 ng/ml ANGPT2, 100 U/ml IFN- γ , and 5 ng/ml IL-4. CD3⁺ cells cultured without monocytes, unlabeled CD3⁺ cells, and cocultures without beads served as experimental controls. Neutralizing anti-*IL-10* (clone 23738; R&D Systems) or IgG control was used at 5 μ g/ml. Following incubation, cells were removed from culture dishes, washed, resuspended in HBSS/0.5% BSA, and

*Academic Unit of Inflammation and Tumour Targeting, University of Sheffield, Sheffield S10 2RX, United Kingdom; [†]Academic Unit of Oral and Maxillofacial Medicine and Surgery, University of Sheffield, Sheffield S10 2RX, United Kingdom; [‡]Institute of Neurology/Edinger Institute, Frankfurt University Medical School, Frankfurt 60528, Germany; and [§]Angiogenesis and Tumor Targeting Research Unit, San Raffaele-Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, 20132 Milan, Italy

Received for publication August 18, 2010. Accepted for publication January 27, 2011.

This work was supported by the Breast Cancer Campaign, U.K. (to C.E.L., C.M., and M.D.P.) and the German Research Foundation (Grant SFB/TR23 [Project C1] to Y.R. and K.H.P.).

Address correspondence and reprint requests to Prof. Claire E. Lewis, Academic Unit of Inflammation and Tumour Targeting, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX, United Kingdom. E-mail address: claire.lewis@sheffield.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: ANGPT2, angiopoietin 2; ARG1, arginase 1; DT, double-transgenic; GCV, gancyclovir; iNOS, inducible NO synthase; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; TEM, TIE2-expressing monocyte/macrophage; Treg, regulatory T cell.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/\$16.00

blocked with an FcR blocking reagent (Miltenyi Biotec) for 10 min at 4°C. Cells were stained with Pacific Blue-conjugated CD14 (1:20, clone M5E2; BD Biosciences), Alexa Fluor 647-conjugated CD3 (1/20, clone OKT3; eBioscience), PE-Cy7-conjugated CD4 (1/20, clone GK1.5; eBioscience), Alexa Fluor 700-conjugated CD8 (1/10, clone OKT8; eBioscience), PE-conjugated CD25 (1/20, clone BC96; eBioscience), or isotype controls for 30 min at 4°C and then washed. Cells were permeabilized using Cytofix/Cytoperm solutions and then stained with PerCP-Cy5.5-conjugated FOXP3 (1/20, clone PCH101; eBioscience) or isotype control. Cells were analyzed on an LSR II flow cytometer (BD Biosciences) using FlowJo software (Tree Star). Compensation was calculated by CompBeads (BD Biosciences), according to the manufacturer's instructions. Experiments were repeated at least three times.

Mice and cell lines

Tie1-tTA-driven human ANGPT2 double-transgenic (DT) mice were generated as described previously (5, 13, 14). ANGPT2 DT and wild-type (WT) control mice were depleted of doxycycline 2 wk before implantation of tumor cells. Syngeneic Lewis lung carcinoma cells (2×10^6) were inoculated s.c. and allowed to propagate for 2 wk. Animals were cared for in accordance with German Legislation on the Care and Use of Laboratory Animals.

To generate tumors without TEMs, syngeneic N202.1A mammary tumor cells were injected into FVB mice (Charles River Laboratories) who had received bone marrow transplantation from FVB/*Tie2-*tk** transgenic mice (2). Mice were then given gancyclovir (GCV) to specifically deplete TEMs or PBS as control. Tumors were harvested after 3 wk. All procedures were conducted in accordance with the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (IACUC 324,335) and communicated to the Ministry of Health in Italy and local authorities in Milan.

MMTV-PyMT mice on the FVB/*n-Tg* background were a gift from Z. Werb (University of California, San Francisco, San Francisco, CA). Late-stage mammary tumors were excised at 11–12 wk. 4T1 cells were maintained as described previously (15). BALB/c mice (Harlan Laboratories) at 5 wk old were injected s.c. with 10^6 4T1 mammary carcinoma cells. Animal experiments were conducted in accordance with U.K. Home Office regulations and were approved by the University of Sheffield Ethical Committee.

Immunofluorescence confocal microscopy

Detection of tumor-associated T cells in frozen tumor sections was performed using eFluor660-conjugated anti-CD3 (1/80, clone 17A2), PE-conjugated anti-CD4 (1/160, clone GK1.5), and FITC-conjugated anti-FOXP3 (1/50, clone FJK-16s) or isotype controls all purchased from eBioscience. Nuclei were highlighted using 30 nM DAPI (Invitrogen). Images were captured by using a Zeiss LSM 510 laser scanning confocal microscope. Further details about microscopy and experimental procedure can be found in Ref. 5.

Flow cytometry and FACS of murine cells

PyMT and 4T1 tumors were digested with 1 mg/ml collagenase type IV (Sigma-Aldrich) in HBSS for 1 h at 37°C in an orbital shaker. Cells were washed in HBSS containing 5% FBS twice and then resuspended in HBSS containing 0.5% BSA. After blocking in 10% goat serum (Vector Laboratories) for 10 min at 4°C, cells were incubated with FITC-conjugated F4/80 (1/25, clone CI:A3-1; AbD Serotec), PE-conjugated anti-murine TIE2 (1/50; eBioscience), Alexa Fluor 700-conjugated CD11b (1/50; clone M1/70; eBioscience), FITC-conjugated anti-FOXP3 (1/50, clone FJK-16s eBioscience), PE-conjugated anti-CD4 (1/100; clone GK1.5; eBioscience), Alexa Fluor 700-conjugated CD3 (1/100; clone 17A2; eBioscience), or isotype controls for 30 min at 4°C and then washed. Cells were permeabilized using Cytofix/Cytoperm solutions. Alexa Fluor 647-conjugated IL-10 (1/50, clone JES5-16E3; eBioscience) or isotype control was added for 30 min at 4°C. Cells were analyzed on an LSR II flow cytometer using FlowJo software.

For FACS, 4T1 single-cell suspensions were stained with anti-F4/80 and anti-TIE2 as described above. F4/80⁺TIE2⁻ tumor-associated macrophages (TAMs) and F4/80⁺TIE2⁺ TEMs were isolated as shown in Supplemental Fig. 1 by using BD FACSAria (BD Biosciences). Splens from tumor-bearing animals were stained with PE-conjugated anti-CD3 (eBioscience), anti-CD11b and anti-Gr1-Alexa Fluor 350 (1/10; BD Biosciences). TO-PRO-3 (Invitrogen) was used to measure viability. Myeloid cell subpopulations and CFSE-labeled splenic T cells were cocultured for 3 d in the presence of mouse CD3/CD28 Dynabeads (Invitrogen) and 500 ng/ml anti-IL-10 (clone JES052A5; R&D Systems) or control IgG where indicated. After coculture, cell death and CFSE fluorescence were measured using LSR II cytometer and analyzed with FlowJo software.

Western blot analysis

4T1 tumors were homogenized in T-PER protein extraction buffer (Pierce) with protease inhibitors and EDTA. Protein concentration was determined using the bicinchoninic acid method. A 12% SDS-polyacrylamide gel was loaded with 40 µg protein for separation and then transferred to nitrocellulose. The membrane was blocked with 5% milk protein in TBS-0.2% Tween 20. Anti-ANGPT2 (1/500; Calbiochem) or anti-IL-10 (1/500; R&D Systems) was incubated with the membrane overnight at 4°C and then washed four times in TBST. Anti-rabbit or anti-rat Abs conjugated with HRP were incubated for 1 h at room temperature. Bands were visualized by ECL. Stripped blots were probed with β-actin (1/5000; Sigma-Aldrich). At least two tumors were analyzed for every time point.

Statistical analysis

One-way ANOVA, followed by Newman-Keuls post test, was used to determine *p* values by using GraphPad Prism software. A *p* value <0.05 was considered statistically significant. All data shown are means ± SEs.

Results

ANGPT2-treated human TEMs suppress T cell proliferation

Because our previous studies showed that ANGPT2 upregulates IL-10 in TEMs (5), we investigated whether ANGPT2 can convert

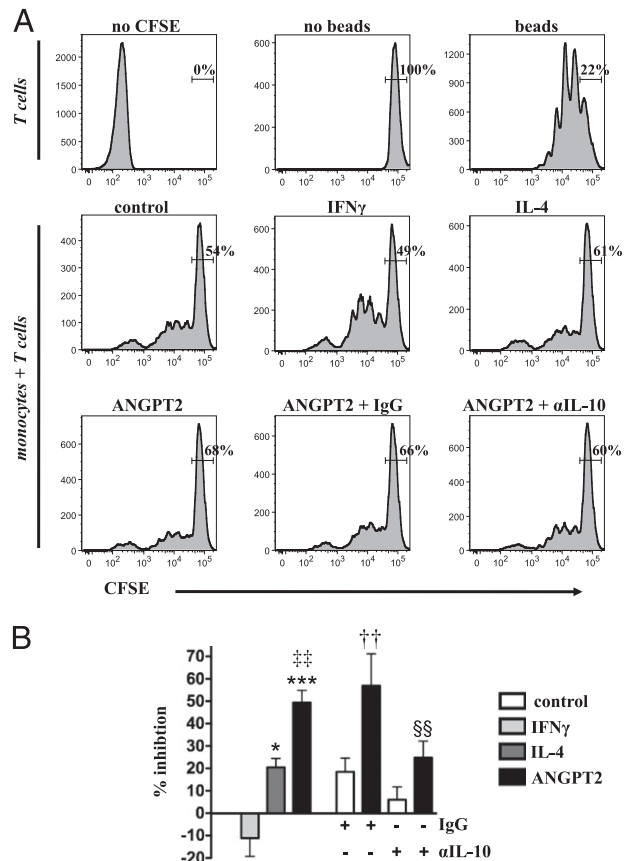


FIGURE 1. ANGPT2-stimulated human TEMs suppress T cell proliferation. Human CD14⁺ monocytes were cocultured with CFSE-labeled CD3⁺ T cells in the presence of CD3/CD28 Dynabeads, 300 ng/ml ANGPT2, 100 U/ml IFN- γ , 5 ng/ml IL-4, or medium alone (control). Cocultures were incubated with a nonspecific IgG or neutralizing IL-10 Ab where indicated. After 5 d, CFSE fluorescence was assessed by flow cytometry on CD14⁺ CD3⁺-gated cells. *A*, Representative histograms of T cell proliferation with percentages of the gated cells shown. *B*, Graphic representation of the change in percentage of gated cells from *A* (expressed as percentage of control). Pooled data from six independent experiments are shown. **p* < 0.05, ****p* < 0.001 as compared with control, †††*p* < 0.01 as compared with IL-4, ††*p* < 0.01 as compared with control + IgG, §§*p* < 0.01 as compared with ANGPT2 + IgG.

these cells into immunosuppressive cells. Human monocytes and autologous CD3⁺ lymphocytes were cocultured in the presence of ANGPT2 or medium alone. IFN- γ and IL-4 were used as controls in coculture assays as they induce an M1/immunostimulatory and M2/immunosuppressive phenotype in monocytes, respectively (9, 10, 16). Monocytes in medium alone had some suppressive function corroborating results from other laboratories (17), and this was significantly enhanced by exposure to the type II/Th2 cytokine IL-4 (Fig. 1). Treatment of cocultures with the type I/Th1 cytokine IFN- γ increased T cell proliferation. Strikingly, ANGPT2-treated TEMs not only suppressed T cell proliferation when compared with control but also its effect was significantly greater than that seen with IL-4 (Fig. 1B). Proliferation of CD3⁺ T lymphocytes cultured without monocytes was not inhibited by the cytokines tested (data not shown). TIE2 receptor expression was never detected on cultured T cells or during coculture with monocytes (data not shown). Moreover, the suppressive effect of ANGPT2-treated TEMs was not due to increased differentiation of the monocytes into macrophages, because CD14 expression on ANGPT2-treated cells was significantly greater than control cells (data not shown).

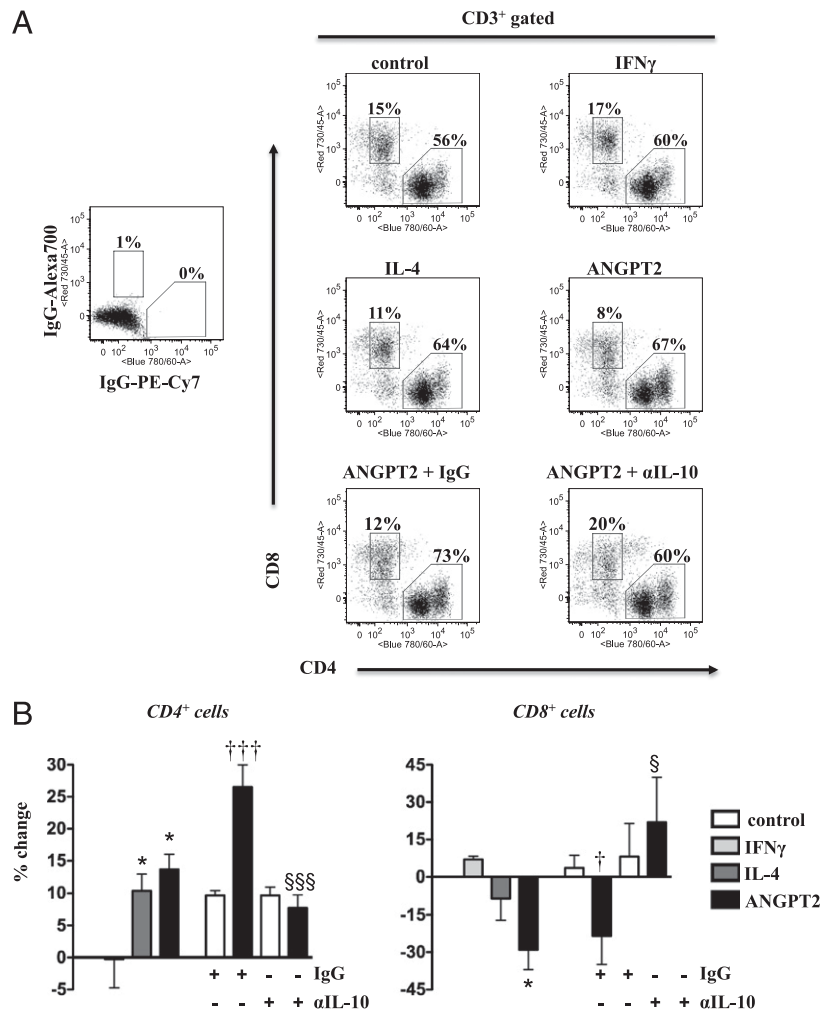
Addition of an IL-10-neutralizing Ab, but not a control Ab, significantly reversed the suppressive functions of ANGPT2-treated monocytes (Fig. 1B), indicating that ANGPT2-induced IL-10 mediates the suppressive ability of TEMs. However, TEM-T cell contact was also required, because inhibition of ANGPT2-induced IL-10 in coculture experiments using Transwell inserts did not significantly reduce T cell suppression (data not shown).

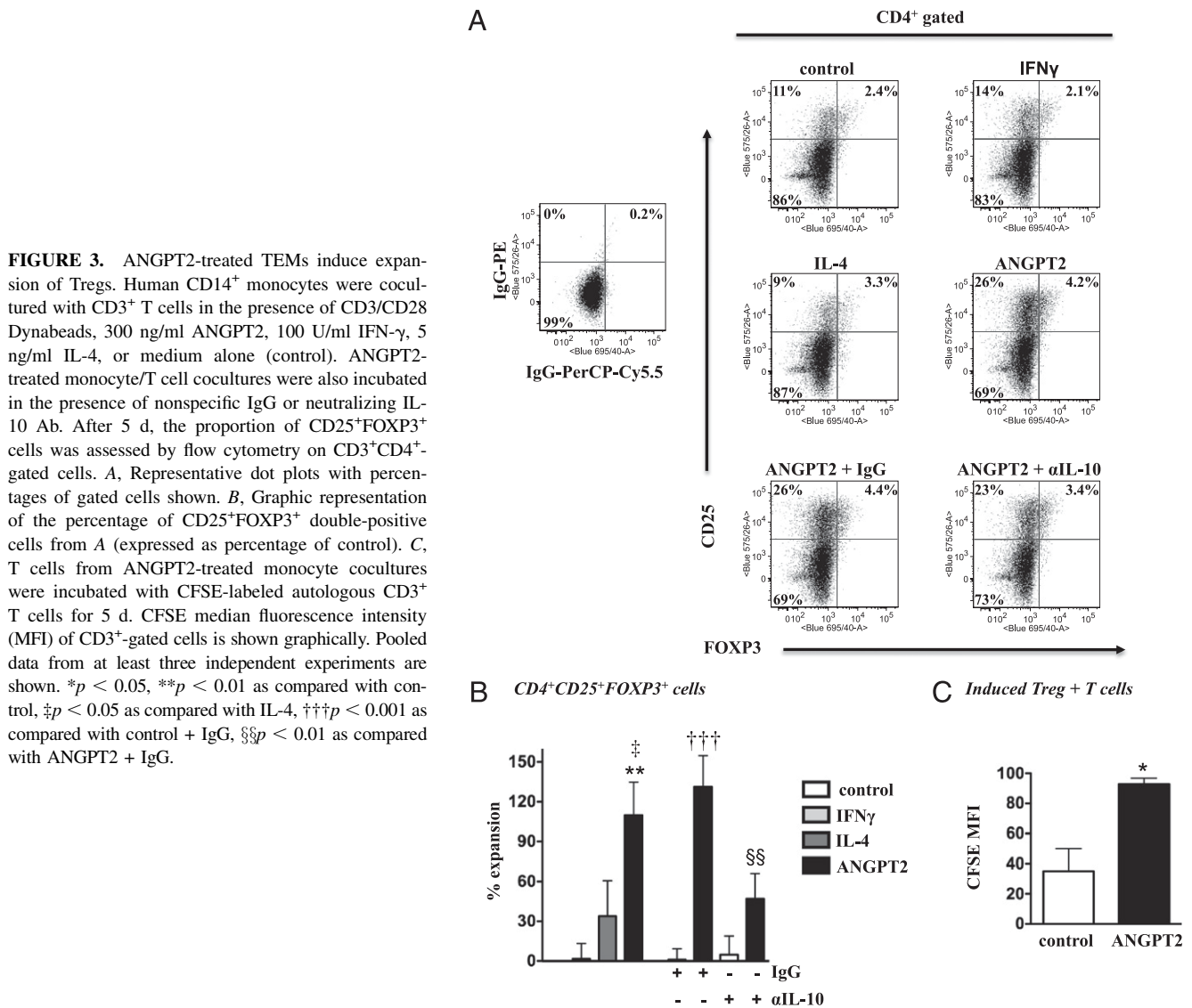
Treg expansion is induced by human TEMs exposed to ANGPT2

We next investigated individual T cell populations in our lymphocyte/monocyte cocultures to determine whether they were affected by cytokine-treated monocytes. We gated on CD4⁺ and CD8⁺ cells and calculated their frequency. The percentage of CD4⁺ cells significantly increased, and CD8⁺ cells decreased with ANGPT2 treatment when compared with control (Fig. 2). Interestingly, ANGPT2 induced a prominent CD3⁺CD4^{high} T cell population that was not observed with IFN- γ , IL-4, or control groups. Moreover, neutralization of ANGPT2-induced IL-10 reversed this ratio back toward control frequencies. These data suggest that ANGPT2 skews the ratio of CD8⁺ cytotoxic T lymphocytes to CD4⁺ cells in favor of the latter.

We then gated on CD4⁺ T cells and analyzed expression of CD25 and FOXP3—two markers of Tregs (17, 18). In the ANGPT2-treated group, CD4⁺CD25^{high}FOXP3⁻ cells were significantly more abundant than control, indicating an increased activation status of the CD4⁺ population (Fig. 3A). In addition, ANGPT2-treated TEMs induced significant expansion of CD4⁺CD25⁺FOXP3⁺ cells when compared with control and IL-4-treated TEMs. IFN- γ and IL-4 treatment had no effect when compared with control (Fig. 3A, 3B). Monocytes were required for expansion of CD4⁺CD25⁺FOXP3⁺ cells, because cytokines alone did not affect the T cells (data not shown). The expansion of CD4⁺CD25⁺FOXP3⁺ cells by ANGPT2-treated TEMs was inhibited by neutralization of IL-10 but not by control Abs, underscoring the functional importance of this cytokine in the generation of Tregs.

FIGURE 2. TEMs exposed to ANGPT2 increase CD4⁺ T cell frequency. Human CD14⁺ monocytes were cocultured with CFSE-labeled CD3⁺ T cells in the presence of CD3/CD28 Dynabeads, 300 ng/ml ANGPT2, 100 U/ml IFN- γ , 5 ng/ml IL-4, or medium alone (control). ANGPT2-treated monocyte/T cell cocultures were also incubated in the presence of nonspecific IgG or neutralizing IL-10 Ab. After 5 d, the proportion of T cell subpopulations was assessed by flow cytometry on CD14⁻CD3⁺-gated cells. **A**, Representative dot plots with percentages of the gated cells shown. **B**, Graphic representation of the percentage of gated cells from **A** (expressed as percentage of control). Pooled data from six independent experiments are shown. * $p < 0.05$ as compared with control; † $p < 0.05$, †† $p < 0.001$ as compared with control + IgG, § $p < 0.05$, §§§ $p < 0.001$ as compared with ANGPT2 + IgG.





After coculture with ANGPT2-treated and control monocytes, the nonadherent T cells were added to CFSE-labeled, autologous CD3⁺ T cells and incubated for another 5 d. The cells induced by ANGPT2-treated TEMs significantly suppressed T cell proliferation when compared with control (Fig. 3C). These data indicate that the Tregs expanded by ANGPT2-treated TEMs exhibit suppressive properties.

Tregs are more abundant in ANGPT2-overexpressing tumors

Given the observations above, we next investigated whether exposure of tumor TEMs to high levels of ANGPT2 in the tumor microenvironment correlates with increased numbers of tumor Tregs. Tumors were propagated in ANGPT2 DT or WT mice as before (5, 14), and then, Tregs were identified as CD3⁺CD4⁺FOXP3⁺ cells. As shown in Fig. 4A and 4B, both the total number of Tregs and the proportion of FOXP3⁺ cells in the CD3⁺CD4⁺ population were significantly increased in ANGPT2 DT tumors when compared with WT tumors (Fig. 4A, 4B). This observation was not due to the general infiltration of T cells, because neither the number of CD3⁺ T cells nor the number of CD3⁺CD4⁺ cells was significantly different between tumor types (Fig. 4B). The proportion of CD3⁺CD4⁺ cells was also similar between tumors overexpressing ANGPT2 and WT tumors (Fig. 4B).

Tregs are reduced in murine mammary tumors following genetic depletion of TEMs

We then examined the effect of TEM depletion on the number of Tregs in N202 mammary tumors by using the conditional model described previously (2). In tumors without TEMs (GCV), there was a trend toward greater CD3⁺ T cell infiltration (Fig. 4D). Both the total number of CD4⁺ T cells and the percentage of CD4⁺ cells among the entire CD3⁺ population were significantly increased, indicative of an antitumor immune response (Fig. 4D). The total number of CD3⁺CD4⁺FOXP3⁺ cells did not significantly differ between tumors with TEMs (PBS) and without TEMs (GCV) (Fig. 4C, 4D). However, the proportion of FOXP3-expressing CD3⁺CD4⁺ cells was significantly decreased in TEM-depleted tumors, suggesting that TEMs suppress antitumor immunity by expanding or recruiting the Treg population into tumors.

Tumor TEMs express higher levels of IL-10 than TIE2⁻ TAMs

F4/80⁺TIE2⁻ TAMs and F4/80⁺TIE2⁺ TEMs were analyzed for expression of intracellular IL-10 by flow cytometry in two different mammary tumor models: the spontaneous MMTV-PyMT and syngeneic 4T1 models. CD11b⁺Gr1⁺ cells from the spleens were also analyzed. In both tumor models, TEMs were the main

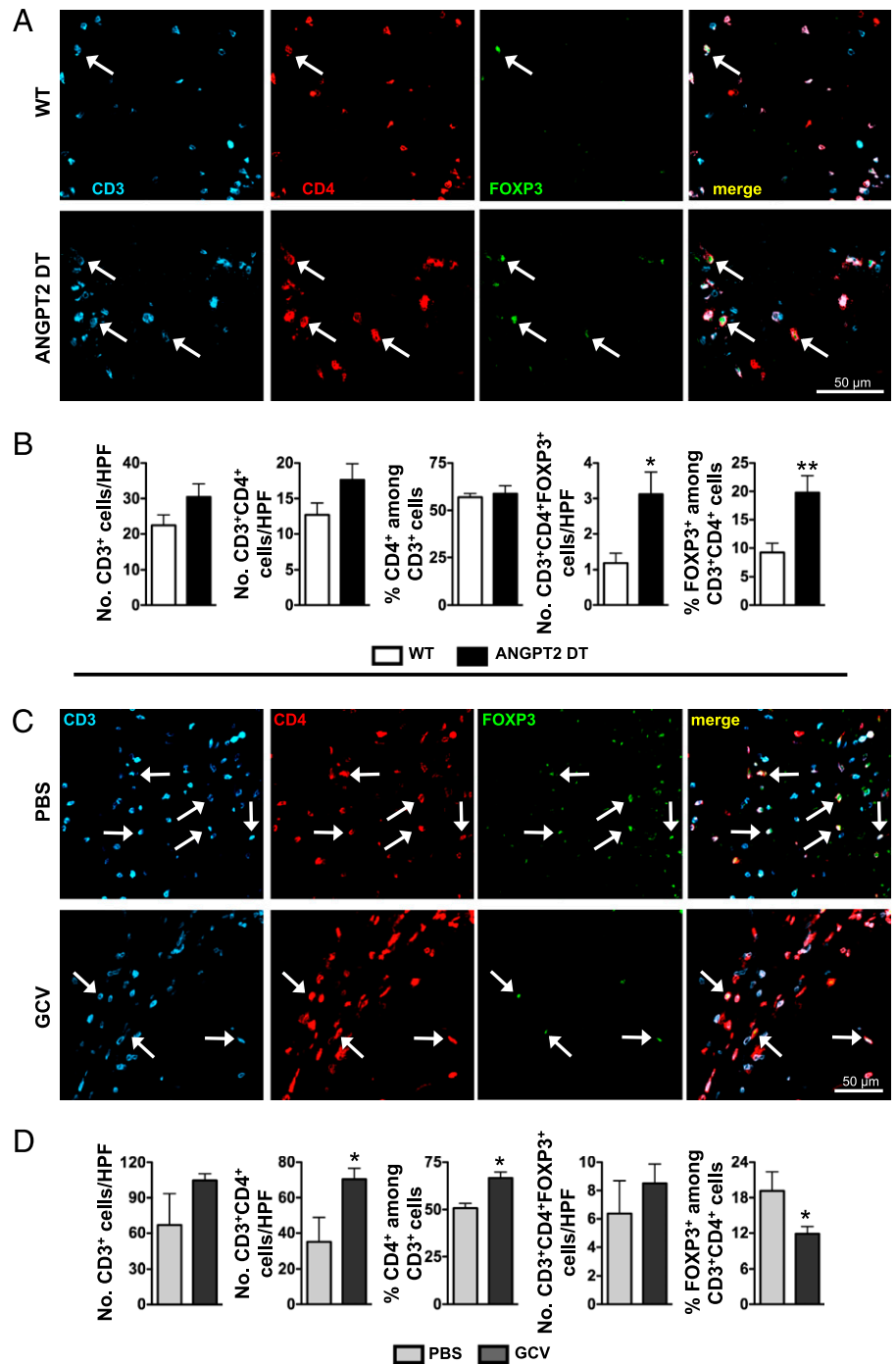


FIGURE 4. Treg frequency correlates with ANGPT2 levels and TEM abundance in tumors. *A* and *B*, Effect of ANGPT2 overexpression: Lewis lung carcinoma cells were inoculated s.c. into *TIE1*-tTA-driven ANGPT2 DT mice then excised after 2 wk (5, 14). *C* and *D*, Effect of TEM depletion: bone marrow from *Tie2*-thymidine kinase (tk) transgenic mice was infused into sublethally irradiated FVB mice. After 6 wk, N202 mammary tumor cells were injected s.c., and tumors were allowed to establish for 2 wk before treatment with GCV to deplete TEMs. PBS- and GCV-treated mice were sacrificed after 3 wk (2). Frozen tumor sections were stained with anti-CD3 (blue), -CD4 (red), and -FOXP3 (green), and representative images are shown. FOXP3-expressing CD3⁺CD4⁺ T cells are denoted by white arrows. The number of cells per high-powered field (HPF) and the proportion of cells among whole populations are represented graphically. **p* < 0.05, ***p* < 0.01.

source of IL-10 among the total F4/80⁺ macrophage population, and IL-10 expression by TEMs was comparable to CD11b⁺Gr1⁺ cells (Fig. 5A). These data suggest that in mammary tumors—where ANGPT2 is upregulated (6–8)—TEMs are stimulated to express high levels of IL-10 to suppress antitumor immunity and to expand the Treg population.

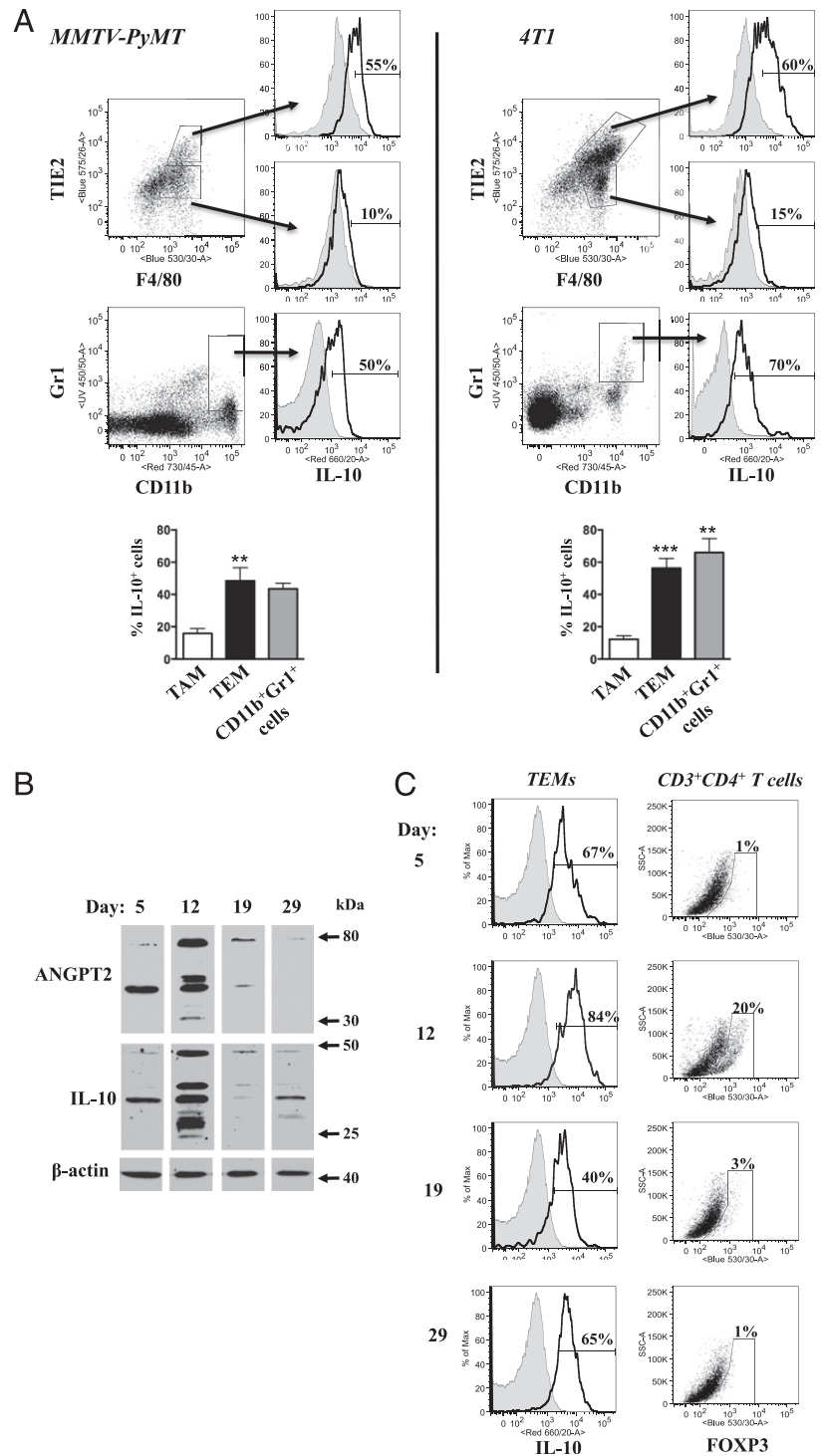
ANGPT2 expression in tumors upregulates TEM-derived IL-10 and Treg infiltration

We measured ANGPT2 expression in the 4T1 tumor model by Western blot over the course of several weeks after tumor cell injection (day 0). Unexpectedly, we observed a dynamic, not constitutive, pattern of ANGPT2 expression (Fig. 5B). Several isoforms of ANGPT2 were detected including the unglycosylated (55 kDa) and glycosylated forms (70 kDa) (19). Lysates from

tumor tissue displayed high levels of ANGPT2 during the early days of tumor growth and then decreased as tumors became larger. Interestingly, IL-10 expression from the same tumor lysates followed a similar dynamic pattern to ANGPT2 expression. IL-10 was also upregulated during the early days of tumor growth (Fig. 5B).

We then quantified intracellular IL-10 in TEMs to determine whether increased levels of ANGPT2 induce TEMs to upregulate IL-10. Tumors from various days posttumor cell injection were enzymatically digested and analyzed by flow cytometry. As expected, F4/80⁺TIE2⁺ TEMs displayed increased IL-10 expression during the early time points when ANGPT2 levels were highest (Fig. 5C). We then hypothesized that TEM-derived IL-10 may influence Treg prevalence in tumors. Strikingly, the proportion of tumor CD3⁺CD4⁺FOXP3⁺ Tregs was greatest on day 12 mim-

FIGURE 5. ANGPT2 expression levels are associated with IL-10 production by TEMs and tumor-infiltrating Tregs. **A**, MMTV-PyMT and 4T1 tumors were excised, enzymatically dispersed, stained with F4/80-FITC, TIE2-PE, IL-10-allophycocyanin, CD11b-Alexa 700, or control Abs, and analyzed by flow cytometry. Gates were placed around CD11b⁺ cells and macrophage subpopulations were identified as F4/80⁺TIE2⁻ TAMs or F4/80⁺TIE2⁺ TEMs. CD11b⁺Gr1⁺ cells were identified in spleens of tumor-bearing mice. Representative histograms are shown of IL-10 expression. Gray peaks represent fluorescence minus one allophycocyanin isotype control. IL-10 expression is also represented graphically. ***p* < 0.01, ****p* < 0.001 as compared with TAMs. **B**, Western blot analysis of ANGPT2 and IL-10 expression in 4T1 whole tumor lysates at various days posttumor cell injection (day 0). β -Actin was used as a loading control. **C**, Histograms of IL-10 expression by TEMs and dot plots of FOXP3 expression from CD3⁺CD4⁺-gated cells in 4T1 tumors with percentage of positive cells displayed above the gate.



icking ANGPT2 expression patterns and upregulation of IL-10 by TEMs (Fig. 5C). On day 12, the frequency of FOXP3-expressing CD3⁺CD4⁺ cells increased ~10-fold when compared with other days. These data provide a direct link between ANGPT2 expression, IL-10 production by TEMs, and Treg abundance in tumors.

Tumor TEMs are immunosuppressive ex vivo

To determine whether TEMs in tumors modulate T cell behavior, we isolated F4/80⁺TIE2⁻ TAMs and F4/80⁺TIE2⁺ TEMs from 4T1 tumors as well as splenic CD11b⁺Gr1⁺ cells by FACS (Supplemental Fig. 1). Myeloid subpopulations were then incubated

with CFSE-labeled CD3⁺ T cells ex vivo. TEMs and CD11b⁺Gr1⁺ cells exhibited a significantly greater ability to inhibit the proliferation of T cells when compared with TIE2⁻ TAMs (Fig. 6). Blocking IL-10 activity with Abs reduced the suppressive effect of TEMs and CD11b⁺Gr1⁺ cells, suggesting that IL-10 mediates the ability of TEMs to prevent T cell proliferation.

Discussion

To our knowledge, this study shows for the first time that ANGPT2 stimulates human TEMs to markedly suppress T cell proliferation. We previously reported that the immunosuppressive cytokine IL-10 is upregulated by TEMs in response to ANGPT2 stimulation

(5), and we demonstrate in this study that TEMs are the predominant source of IL-10 among all F4/80⁺ macrophages in two different mammary tumor models. Moreover, ANGPT2-induced, TEM-derived IL-10 suppressed T cell proliferation and expanded CD4⁺CD25^{high}FOXP3⁺ Tregs in vitro. These data indicate that TEMs promote tumor progression not only by angiogenic stimulation but also through subversion of antitumor immunity and induction of tolerance via expansion of Tregs.

We chose to use mixed cultures of both TIE2⁻ and TIE⁺ human monocytes in our cocultures, because this represents the monocyte heterogeneity present in peripheral blood and tumors. Because TIE2⁻ monocytes do not respond to ANGPT2 (4, 5), the effects elicited by ANGPT2 in our cocultures represent the responses of TEMs to this cytokine.

The main source of ANGPT2 in tumors is endothelial cells (6–8), so newly recruited TEMs undergoing diapedesis likely encounter high levels of ANGPT2 as they enter the tumor. ANGPT2 may then skew TEM phenotype toward a more M2-like polarization through upregulation of proangiogenic and immunosuppressive genes (3, 5, 11). Our data indicate that ANGPT2 is critical for the suppressive ability of TEMs as well as their expression of IL-10, and this notion is supported by several observations presented in this study. The varied expression levels of ANGPT2 as tumors progressed and expanded surprised us. However, publications by other groups using ANGPT2 knockout mice have shown that ANGPT2 is essential during the early stages of tumorigenesis but dispensable for later stages (20). Our data corroborate their findings and suggest that ANGPT2 helps to establish the initial immunosuppressive environment via upregulation of IL-10 by TEMs and Treg expansion.

In addition to IL-10, other macrophage-derived cytokines and enzymes have been implicated in suppression of T cell proliferation including TGF- β , cyclooxygenase-2-induced PGE₂, inducible NO synthase (iNOS)2, and arginase 1 (ARG1) (10, 12, 18, 21–23). Pucci et al. (11) reported that tumor TEMs express higher levels of *Arg1* and lower levels of *Tgf β* , *Cox-2*, and *Nos2* mRNA than TIE2⁻ TAMs, although differences at the protein level for these molecules have yet to be confirmed. It is possible that ANGPT2 also stimulates the expression of one or more of these immunosuppressive factors by TEMs. *Il-10* mRNA expression levels did not differ between TEMs and TAMs (11), whereas we found TEMs expressed significantly more IL-10 protein than TAMs. This is not altogether surprising considering IL-10 is constitutively transcribed in many cell types and highly dependent on posttranscriptional regulation (24–27).

Myeloid-derived suppressor cells (MDSCs) also produce IL-10 and ARG1 (18, 21–23). However, TEMs and the classical CD11b⁺Gr1⁺ MDSCs do not appear to be overlapping populations. TEMs do not express Gr1—a commonly used marker to identify MDSCs in secondary lymphoid organs and blood of tumor-bearing mice as well as circulating inflammatory monocytes (18, 22). Rather, TEMs share a related gene signature with circulating Gr1⁻ resident monocytes (1, 2, 11), and splenic CD11b⁺Gr1⁺ MDSCs from lymphoma-bearing mice fail to express TIE2 (22). It remains to be seen whether TEMs in tumors and MDSCs in distant, secondary lymphoid organs work in concert to suppress antitumor immune responses. In addition, TEMs can now be classified as MDSCs, because the heterogeneous myeloid cells within this group are defined by their functional ability to inhibit T cell proliferation, rather than their surface marker expression (28, 29).

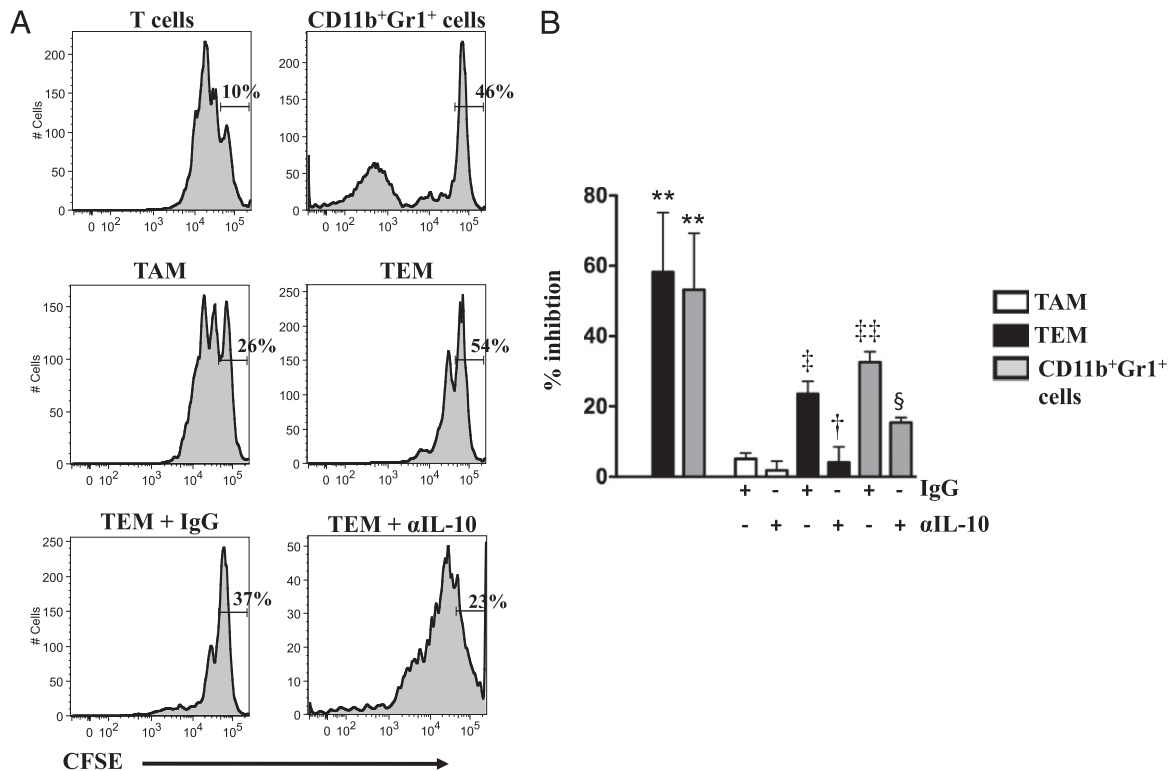


FIGURE 6. Tumor TEMs inhibit T cell proliferation. F4/80⁺TIE2⁻ TAMs and F4/80⁺TIE2⁺ TEMs from 4T1 tumors as well as CD11b⁺Gr1⁺ cells and CD3⁺ from spleens were isolated by FACS. Myeloid cell subpopulations were cultured with CFSE-labeled CD3⁺ T cells for 3 d. **A**, Representative histograms of T cell proliferation following incubation with percentages of the gated cells shown. **B**, Graphic representation of the percentage of gated cells from **A** (expressed as percentage of TAM group). Combined data from four independent experiments are shown. ***p* < 0.01 as compared with TAMs, ‡*p* < 0.05, ††*p* < 0.01 as compared with TAM + IgG, †*p* < 0.05 as compared with ANGPT2 + IgG, §*p* < 0.05 as compared with CD11b⁺Gr1⁺ cells + IgG.

Although TEMs and classic MDSCs are likely different myeloid cell populations, a recent publication characterizing CD11b⁺F4/80⁺ TAMs as MHC-II^{high} and MHC-II^{low} subsets indicate that TEMs make up a fraction of the MHC-II^{low} cell population (30). In fact, the gene signature, phenotype, and superior proangiogenic abilities of TEMs and MHC-II^{low} TAMs are analogous (11, 30). Interestingly, Ag processing by MHC-II^{low} cells is less efficient than MHC-II^{high} cells, but both subpopulations inhibit T cell proliferation when compared with splenic dendritic cells. MHC-II^{high} and MHC-II^{low} cells are equally capable of suppressing anti-CD3-stimulated T cells with each subset using different mechanisms to accomplish this. MHC-II^{high} cell-mediated suppression is iNOS dependent, whereas the molecule(s) used by MHC-II^{low} cells is unknown. These data, together with the data presented in this paper, suggest that TEMs use IL-10, not ARG1 or iNOS, to inhibit T cell proliferation. However, the equivalent immunosuppressive potential of MHC-II^{high} and MHC-II^{low} cells contrasts with our own data showing that TEMs possess significantly greater ability to suppress T cell proliferation than TIE2⁻ TAMs. Varying levels of ANGPT2 in different tumor models may explain this discrepancy, because ANGPT2 may drive TEMs' T cell-inhibitory functions. Moreover, the shaping of different T cell populations (i.e., expansion of Tregs by TEMs) in the tumor microenvironment may be more important to tumor progression than inhibiting T cell proliferation (30).

In summary, we demonstrate a novel tumor-promoting function for TEMs: the ability to suppress T cell proliferation and expand Tregs via ANGPT2-induced IL-10. These data suggest that inhibition of the ANGPT2–TIE2 interaction in tumors could impede angiogenesis and reinitiate antitumor immunity (31).

Acknowledgments

We thank Dr. Zena Werb for the MMTV-PyMT mice, Dr. Dan Dumont for the ANGPT2 DT mice, and Susan Newton and Kay Hopkinson (University of Sheffield Flow Cytometry Core Facility).

Disclosures

The authors have no financial conflicts of interest.

References

- De Palma, M., M. A. Venneri, C. Roca, and L. Naldini. 2003. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* 9: 789–795.
- De Palma, M., M. A. Venneri, R. Galli, L. Sergi, L. S. Politi, M. Sampaolesi, and L. Naldini. 2005. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 8: 211–226.
- Murdoch, C., S. Tazzyman, S. Webster, and C. E. Lewis. 2007. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *J. Immunol.* 178: 7405–7411.
- Venneri, M. A., M. De Palma, M. Ponzoni, F. Pucci, C. Scielzo, E. Zonari, R. Mazziere, C. Dogliani, and L. Naldini. 2007. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood* 109: 5276–5285.
- Coffelt, S. B., A. O. Tal, A. Scholz, M. De Palma, S. Patel, C. Urbich, S. K. Biswas, C. Murdoch, K. H. Plate, Y. Reiss, and C. E. Lewis. 2010. Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer Res.* 70: 5270–5280.
- Sfiligoi, C., A. de Luca, I. Cascone, V. Sorbello, L. Fusco, R. Ponzzone, N. Biglia, E. Audero, R. Arisio, F. Bussolino, et al. 2003. Angiopoietin-2 expression in breast cancer correlates with lymph node invasion and short survival. *Int. J. Cancer* 103: 466–474.
- Tsutsui, S., H. Inoue, K. Yasuda, K. Suzuki, H. Takeuchi, T. Nishizaki, H. Higashi, S. Era, and M. Mori. 2006. Angiopoietin 2 expression in invasive ductal carcinoma of the breast: its relationship to the VEGF expression and microvessel density. *Breast Cancer Res. Treat.* 98: 261–266.
- Augustin, H. G., G. Y. Koh, G. Thurston, and K. Alitalo. 2009. Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat. Rev. Mol. Cell Biol.* 10: 165–177.
- Mills, C. D., K. Kincaid, J. M. Alt, M. J. Heilman, and A. M. Hill. 2000. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* 164: 6166–6173.
- Mantovani, A., A. Sica, and M. Locati. 2007. New vistas on macrophage differentiation and activation. *Eur. J. Immunol.* 37: 14–16.
- Pucci, F., M. A. Venneri, D. Bizziato, A. Nonis, D. Moi, A. Sica, C. Di Serio, L. Naldini, and M. De Palma. 2009. A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood* 114: 901–914.
- Coffelt, S. B., R. Hughes, and C. E. Lewis. 2009. Tumor-associated macrophages: effectors of angiogenesis and tumor progression. *Biochim. Biophys. Acta* 1796: 11–18.
- Bureau, W., P. Van Slyke, J. Jones, R. N. Han, N. L. Ward, D. J. Stewart, and D. J. Dumont. 2006. Chronic systemic delivery of angiopoietin-2 reveals a possible independent angiogenic effect. *Am. J. Physiol. Heart Circ. Physiol.* 291: H948–H956.
- Reiss, Y., J. Droste, M. Heil, S. Tribulova, M. H. Schmidt, W. Schaper, D. J. Dumont, and K. H. Plate. 2007. Angiopoietin-2 impairs revascularization after limb ischemia. *Circ. Res.* 101: 88–96.
- Ryan, R. M., J. Green, P. J. Williams, S. Tazzyman, S. Hunt, J. H. Harmey, S. C. Kehoe, and C. E. Lewis. 2009. Bacterial delivery of a novel cytotoxin to hypoxic areas of solid tumors. *Gene Ther.* 16: 329–339.
- Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potentially enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176: 287–292.
- Mirza, N., and D. Gabrilovich. 2007. Comment on "Cutting edge: induction of B7-H4 on APCs through IL-10: novel suppressive mode for regulatory T cells". *J. Immunol.* 178: 4705–4706, author reply 4706.
- Huang, B., P. Y. Pan, Q. Li, A. I. Sato, D. E. Levy, J. Bromberg, C. M. Divino, and S. H. Chen. 2006. Gr-1⁺CD115⁺ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res.* 66: 1123–1131.
- Kim, L., J. H. Kim, Y. S. Ryu, S. H. Jung, J. J. Nah, and G. Y. Koh. 2000. Characterization and expression of a novel alternatively spliced human angiopoietin-2. *J. Biol. Chem.* 275: 18550–18556.
- Nassar, P., M. Thomas, K. Kruse, I. Helfrich, V. Wolter, C. Deppermann, D. Schadendorf, G. Thurston, U. Fiedler, and H. G. Augustin. 2009. Host-derived angiopoietin-2 affects early stages of tumor development and vessel maturation but is dispensable for later stages of tumor growth. *Cancer Res.* 69: 1324–1333.
- Bronte, V., P. Serafini, C. De Santo, I. Marigo, V. Tosello, A. Mazzoni, D. M. Segal, C. Staib, M. Lowel, G. Sutter, et al. 2003. IL-4-induced arginase 1 suppresses alloreactive T cells in tumor-bearing mice. *J. Immunol.* 170: 270–278.
- Movahedi, K., M. Guillemins, J. Van den Bossche, R. Van den Bergh, C. Gysemans, A. Beschinn, P. De Baetselier, and J. A. Van Ginderachter. 2008. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. *Blood* 111: 4233–4244.
- Rodríguez, P. C., D. G. Quiceno, J. Zabaleta, B. Ortiz, A. H. Zea, M. B. Piazuelo, A. Delgado, P. Correa, J. Brayer, E. M. Sotomayor, et al. 2004. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 64: 5839–5849.
- Ma, W., W. Lim, K. Gee, S. Aucoin, D. Nandan, M. Kozlowski, F. Diaz-Mitoma, and A. Kumar. 2001. The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages. *J. Biol. Chem.* 276: 13664–13674.
- Németh, Z. H., C. S. Lutz, B. Csóka, E. A. Deitch, S. J. Leibovich, W. C. Gause, M. Tone, P. Pacher, E. S. Vizi, and G. Haskó. 2005. Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated post-transcriptional mechanism. *J. Immunol.* 175: 8260–8270.
- Sharma, A., M. Kumar, J. Aich, M. Hariharan, S. K. Brahmachari, A. Agrawal, and B. Ghosh. 2009. Posttranscriptional regulation of interleukin-10 expression by hsa-miR-106a. *Proc. Natl. Acad. Sci. USA* 106: 5761–5766.
- Tone, M., M. J. Powell, Y. Tone, S. A. Thompson, and H. Waldmann. 2000. IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. *J. Immunol.* 165: 286–291.
- Gabrilovich, D. I., and S. Nagaraj. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9: 162–174.
- Gabrilovich, D. I., V. Bronte, S. H. Chen, M. P. Colombo, A. Ochoa, S. Ostrand-Rosenberg, and H. Schreiber. 2007. The terminology issue for myeloid-derived suppressor cells. *Cancer Res.* 67: 425, author reply 426.
- Movahedi, K., D. Laoui, C. Gysemans, M. Baeten, G. Stangé, J. Van den Bossche, M. Mack, D. Pipeleers, P. In't Veld, P. De Baetselier, and J. A. Van Ginderachter. 2010. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* 70: 5728–5739.
- Huang, H., A. Bhat, G. Woodnutt, and R. Lappe. 2010. Targeting the ANGPT-TIE2 pathway in malignancy. *Nat. Rev. Cancer* 10: 575–585.