Amphiregulin Enhances Regulatory T Cell-Suppressive Function via the Epidermal Growth Factor Receptor

Dietmar M.W. Zaiss,[1,*] Jorg van Loosdregt,[3,4] Andrea Gorlani,[1,5] Cornelis P.J. Bekker,[1,3] Andrea Gröne,[2] Maria Sibilia,[6] Paul M.P. van Bergen en Henegouwen,[5] Rob C. Roovers,[5] Paul J. Coffer,[3,4] and Alice J.A.M. Sijts[1]
[1]Department of Infectious Diseases & Immunology
[2]Department of Pathobiology
Faculty of Veterinary Medicine, University of Utrecht, 3584 CL Utrecht, the Netherlands
[3]Center for Molecular & Cellular Intervention, Wilhelmina Children's Hospital, University Medical Center Utrecht, 3584 EA Utrecht, the Netherlands
[4]Department of Cell Biology, University Medical Center Utrecht, 3508 AB Utrecht, the Netherlands
[5]Department of Cell Biology, Science Faculty, University of Utrecht, 3584 CH Utrecht, the Netherlands
[6]Institute for Cancer Research, Medical University of Vienna, 1090 Vienna, Austria
*Correspondence: d.zaiss@uu.nl
http://dx.doi.org/10.1016/j.immuni.2012.09.023

SUMMARY

Epidermal growth factor receptor (EGFR) is known to be critically involved in tissue development and homeostasis as well as in the pathogenesis of cancer. Here we showed that Foxp3^{+} regulatory T (Treg) cells express EGFR under inflammatory conditions. Stimulation with the EGF-like growth factor Amphiregulin (AREG) markedly enhanced Treg cell function in vitro, and in a colitis and tumor vaccination model we showed that AREG was critical for efficient Treg cell function in vivo. In addition, mast cell-derived AREG fully restored optimal Treg cell function. These findings reveal EGFR as a component in the regulation of local immune responses and establish a link between mast cells and Treg cells. Targeting of this immune regulatory mechanism may contribute to the therapeutic successes of EGFR-targeting treatments in cancer patients.

INTRODUCTION

Stimulation of the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGFR) induces a complex cascade of phosphorylation and activation events that determine cell-fate decisions, such as proliferation or differentiation, and the development of tumors (Schlessinger, 2000; Avraham and Yarden, 2011). The EGFR is well established to be ubiquitously expressed but, in general, is thought to be absent on the hematopoietic cell linage. This assumption has ignored sporadic demonstrations of EGFR expression on leukocyte populations, such as monocytes (Chan et al., 2009) or plasma cells (Mahtouk et al., 2005). In addition to EGFR, its ligands have also been shown to be produced not only by epithelial cells but also by activated leukocyte and lymphocyte populations. For example, the EGF-like growth factor Amphiregulin (AREG) is expressed by activated Th2 cells (Zaiss et al., 2006), mast cells (Wang et al., 2005; Okumura et al., 2005), eosinophils (Matsumoto et al., 2009), and basophils (Qi et al., 2010). These data

suggest that the EGFR signaling pathway could play a more substantial role within the immune system than so far appreciated.

Hints for such an assumption can also be found from the widespread use of EGFR-targeted treatments in cancer patients. Targeting the EGFR has become an established approach in tumor treatment and such treatments improve the overall and the progression-free survival of patients suffering from different types of cancer, such as colorectal (Cunningham et al., 2004) and non-small-cell lung cancer (NSCLC) (Bonner et al., 2006). The observed clinical successes of this treatment remain poorly understood but are assumed to be predominantly mediated by interference with survival and growth signals needed by the tumor. This concept is supported by a number of findings, such as the demonstration of tumor adaption within a patient in response to treatment (Montagut et al., 2012), suggesting strong evolutionary pressure mediated by treatment on the tumor. Nevertheless, other studies indicate that a substantial part of the clinical responses observed after EGFR targeting treatments may be mediated not only by direct effects on the tumor but also by treatment-induced immune responses (Ferris et al., 2010). For example, Garrido et al. (2007) demonstrated in a Lewis lung cell tumor model that the effect of an EGFR blocking antibody treatment was virtually entirely mediated by treatment-induced T cell responses. Such involvement of treatment-induced immune responses may also explain a number of somewhat enigmatic clinical reports, such as objective clinical responses to EGFR-targeted treatments from patients with tumors lacking detectable EGFR expression (Chung et al., 2005). Most interestingly, it has been reported that the expression of AREG constitutes the best known predictor for a low therapy efficacy in cancer patients with a tumor expressing a nonmutated form of K-Ras (Tinhofer et al., 2011; Jacobs et al., 2009; Khambata-Ford et al., 2007; Ishikawa et al., 2005).

AREG is known to be a type II cytokine (Zaiss et al., 2006), for which it is established that one of their main function is the control of inflammation (Chen et al., 2012). In light of these findings, we wanted to determine whether AREG may contribute to the regulation of immune responses. This study showed that regulatory T (Treg) cells expressed EGFR under inflammatory conditions and that AREG was of pivotal importance to ensure

A

WT donor - WT recipient   *Areg*⁻/⁻ donor - WT recipient

B

C
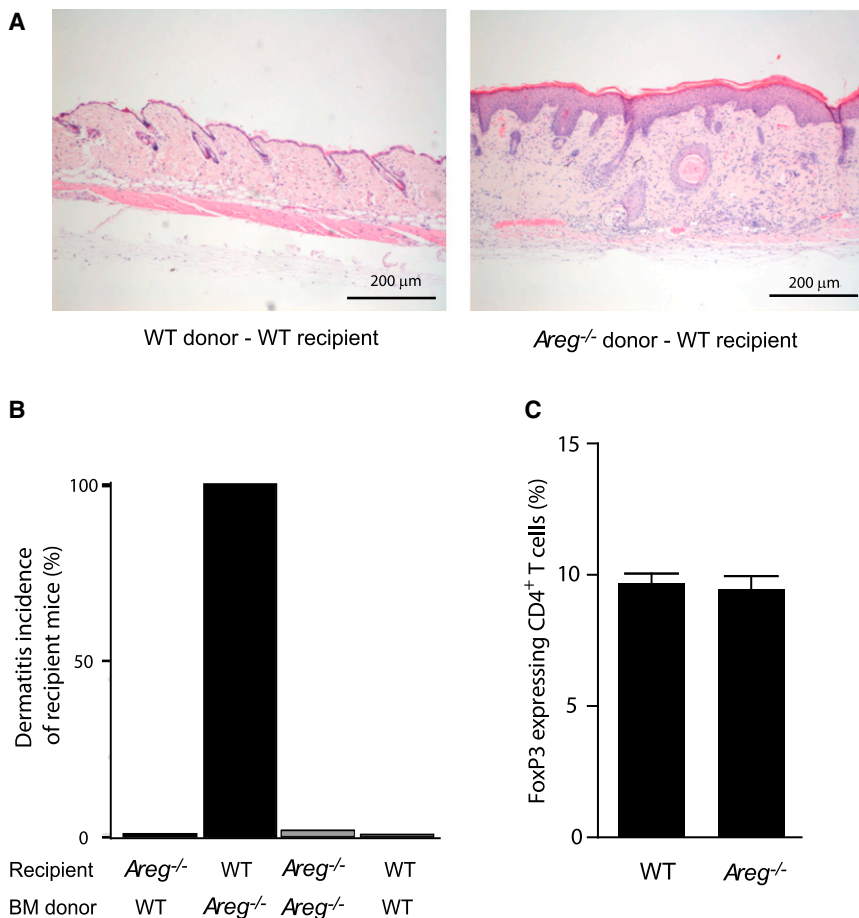
Figure 1. BM Chimeric WT Mice Reconstituted with *Areg*⁻/⁻ BM Develop Dermatitis
(A and B) BL6-SJL (CD45.1) or *Areg*⁻/⁻ mice were irradiated with 10 Gy X rays and reconstituted with 10⁷ BM cells derived from BL6-SJL or *Areg*⁻/⁻ mice. Incidence of dermatitis of recipient mice 6 weeks after BM transfer is shown (n = 4 mice per group, bars represent means + SEM).
(C) Lymphocytes derived from mesenteric lymph nodes of WT or *Areg*⁻/⁻ mice were stained for CD4 and intracellular for FoxP3; frequency of FoxP3-expressing cells is shown (n = 4 mice per group, bars represent means + SEM).
For additional information, see Figure S1.

optimal Treg cell-mediated immune regulation. Thus, our data reveal a mechanism by which Treg cell function is regulated at the site of inflammation.

RESULTS

Amphiregulin Gene-Deficient Mice Display Immune Regulatory Dysfunction

We previously identified the EGF-like growth factor Amphiregulin (AREG) as a type II cytokine (Zaiss et al., 2006). While characterizing AREG gene-deficient (*Areg*⁻/⁻) mice, we noticed an immune regulatory dysfunction in this mouse strain. This was evident, for example, from enhanced frequencies of antigen (Ag)-specific CD4⁺ T cells in *Areg*⁻/⁻ compared with wild-type (WT) C57BL/6 mice after *Listeria monocytogenes* infection (data not shown) and from the development of dermatitis after bone marrow (BM) transplantation of *Areg*⁻/⁻ BM into WT recipient C57BL/6 mice (Figures 1A and 1B). Because FoxP3-expressing Treg cells are of crucial importance for both dampening CD4⁺ T cell responses and immune tolerization after BM transplantation (Cobbold et al., 2006), we postulated that Treg cell function could be impaired in the absence of AREG. Flow cytometry analyses, however, showed similar frequencies of FoxP3-expressing Treg cells in the secondary lymphoid organs of *Areg*⁻/⁻ and WT mice (Figure 1C). Also in the skin-draining, inguinal LN of BM chimeric C57BL/6 WT mice that had received

Areg⁻/⁻ BM, normal frequencies of FoxP3-expressing Treg cells were detected (Figure S1A available online). These data indicate that neither insufficient numbers nor lack of homing of Treg cells to the site of inflammation could explain the lack of immune regulation observed in *Areg*⁻/⁻ mice.

Regulatory T Cells Express the EGFR

To determine whether AREG might have a direct effect on Treg cells, we measured EGFR expression on T cells ex vivo. To this end, we sorted Treg cells from FoxP3-GFP transgenic mice based on GFP expression, as well as Treg cells derived from peripheral blood mononuclear cells (PBMCs) of healthy donors based on high expression of CD25 and the presence or absence of CD127. Analysis by quantitative RT-PCR (qRT-PCR) showed clearly detectable amounts of EGFR mRNA in Treg cells derived from either species (Figures 2A–2C). In human PBMCs, very low EGFR mRNA expression was detectable also in conventional CD4⁺ T cells, but not in CD8⁺ T cells (Figure 2B).

To verify EGFR expression on Treg cells by flow cytometry analysis, we used a biotinylated nanobody specific for a shared region of the mouse and human EGFR (Figure S6). About 15% of the FoxP3- and Helios-expressing CD4⁺ T cells in the peripheral blood of healthy volunteers expressed the EGFR (Figure 2D, for gating strategies see Figure S2A) and very low amounts of EGFR were detectable on FoxP3-expressing CD4⁺ T cells in the spleen of healthy mice (data not shown). B16 melanoma-residential Treg cells, however, expressed well detectable amounts of the EGFR (Figure 2E, for gating strategies see Figure S2B). The staining was specific and absent on FoxP3-expressing CD4⁺ T cells derived from B16 melanoma of *Egfr*ᶠˡᵒˣ/ᶠˡᵒˣ × *Cd4-cre* mice (Figure 2E). Because in humans almost all EGFR-expressing Treg cells were FoxP3ʰⁱ and CD45RA⁻ (Figure S2C), a subtype of Treg cells that Sakaguchi and colleagues described as activated Treg cells (Miyara et al., 2009), and because human Treg cells gained EGFR expression upon in vitro activation (data not shown), we concluded that Treg cells express the EGFR upon activation.
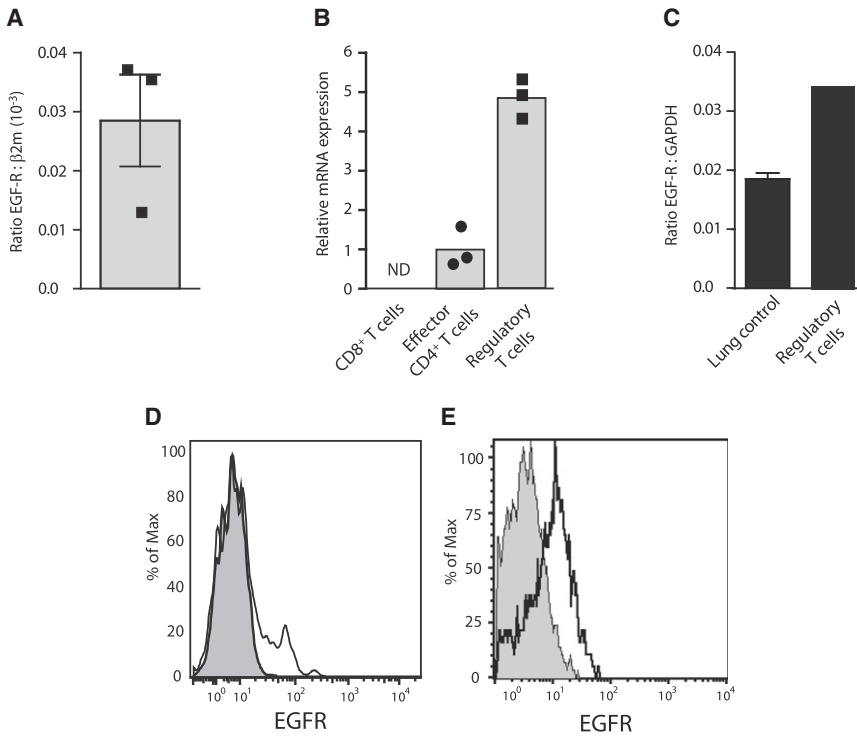
Figure 2. Treg Cells Express EGFR

(A and B) T cells from the blood of healthy volunteers were sorted by flow cytometry based on CD4 and CD8 expression. Effector T cells and Treg cells were further separated based on nonoverlapping expression markers, i.e., CD127^{hi} versus CD127^{lo} and CD25^{lo} versus CD25^{hi}, respectively. EGFR expression was determined by quantitative RT-PCR. Absolute expression in comparison to β2 m expression (A) and relative EGFR expression of different T cell populations to each other (B). n = 3 volunteers per group, bars represent means + SEM. ND, nondetectible (no signal for EGFR).

(C) Treg cells derived from the spleen of FoxP3-GFP mice were sorted by flow cytometry based on GFP expression. EGFR mRNA expression was determined by quantitative RT-PCR (experiment performed in triplicate, bar represents means ± SEM).

(D) PBMCs of healthy volunteers were blocked with an excess of unspecific nanobodies and then stained for CD4 and for EGFR via an EGFR-specific biotinylated nanobody. Thereafter, cells were stained intracellularly for Helios and FoxP3 and SA-PE. Black line shows EGFR staining; filled line represents background SA-PE-only staining.

(E) EGFR expression on tumor-infiltrating Treg cells derived from B16 melanoma of WT C57BL/6 or *Egfr*[flox/flox] × *Cd4-cre* mice was analyzed by flow cytometry. Cells were stained for CD4, EGFR, and FoxP3. Full lines show the staining of Treg cells derived from WT mice, filled lines the staining of Treg cells derived from *Egfr*[flox/flox] × *Cd4-cre* mice. For additional information, see Figure S2.

Amphiregulin Enhances Regulatory T Cell Function

The EGFR and the T cell receptor (TCR) share a common signal transduction pathway, the ERK-MAP-kinase module, and AREG treatment substantially increased ERK activation in differentiated induced Treg cells (Figure 3A). In contrast to in effector T cells, where upon TCR engagement the MAP kinase pathway in a binary manner is briefly activated and then rapidly turned off (Altan-Bonnet and Germain, 2005), this pathway in Treg cells is activated for an extended period of time (Tsang et al., 2006). This situation closely correlated with the MAP kinase signal transduction pathway downstream of the EGFR. Most EGFR ligands, such as EGF or TGF-α, induce a strong but transient signal. Such a signal initiates ubiquitination via the E3-ligase Clb, which then induces rapid internalization and degradation of the EGFR and thus a transient desensitization. AREG ligation on the other hand induces a sustained, tonic signal through the MAP kinase signal transduction pathway, which does not induce internalization and degradation of the EGFR (Stern et al., 2008). Thus, we hypothesized that an AREG-induced signal may support and sustain MAP kinase activation in Treg cells, thereby enhancing their regulatory function.

To address this hypothesis, we determined the effect of AREG on modulating Treg cell function in in vitro suppression assays. As shown in Figures 3B and S3A, the presence of AREG during the assay significantly enhanced the suppressive capacity of Treg cells. Importantly, AREG had no influence on the overall proliferation or survival of Treg cells and did not directly influence the proliferation of effector cells (Figures S3B and S3C). As a control for the specificity of AREG, we performed in vitro suppression assays in the presence of the EGFR-specific tyrosine kinase inhibitor Gefitinib, which entirely eliminated the AREG-mediated effect (Figure 3C).

The effect of AREG on the suppressive activity of Treg cells became more pronounced the more the activating anti-CD3ε was diluted (Figure 3D). Although the dilution of the antibody had no appreciable direct effect on the proliferation of the effector T cells (data not shown), the suppressive capacity of Treg cells substantially declined in the absence but not in the presence of AREG.

Based on these data we concluded that AREG directly enhances the suppressive capacity of Treg cells in vitro, most probably by enhancing and sustaining the TCR-induced signal through the MAP kinase signaling pathway.

Amphiregulin Is Essential for Efficient Regulatory T Cell Function In Vivo

To determine the physiological relevance of AREG expression on Treg cell function in vivo, we tested the suppressive capacity of Treg cells in a T cell transfer colitis model, the gold standard to determine Treg cell function in vivo (Powrie et al., 1994). To this end, we transferred naive CD4+ T cells in the presence or absence of Treg cells into lymphopenic RAG1-deficient (*Rag1*^{−/−}) or *Areg*^{−/−}*Rag1*^{−/−} mice. Colitis development was determined 6 weeks after transfer according to a histological score established by Berg et al. (1996). As shown in Figure 4A, transfer of a fixed number of naive CD4+ T cells together with increasing amounts of Treg cells into either *Rag1*^{−/−} or *Areg*^{−/−}*Rag1*^{−/−} mice decreased the severity of disease in a
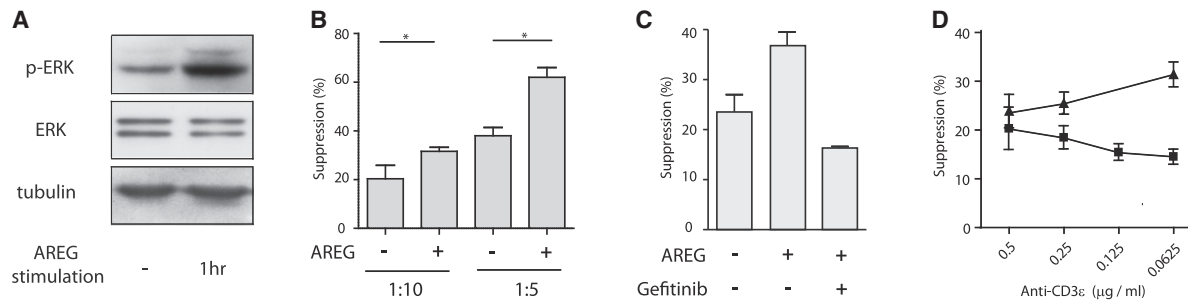
Figure 3. Amphiregulin Enhances the Suppressive Capacity of EGFR-Expressing Treg Cells In Vitro

(A) iTreg cells were differentiated form umbilical cord blood CD4+ T cells in the presence of TGF-β. After 5 days of differentiation, 0.5×10^7 cells were either treated with 100 ng/ml AREG or left untreated and ERK activation determined by protein blot with p-ERK-specific antiserum.

(B and C) PBMCs derived from healthy volunteers were stimulated with membrane-bound anti-CD3 and CD25+CD127−CD4+ Treg cells were added to suppress proliferation of CFSE-labeled CD4+ T cells in the presence or absence of at least 100 ng/ml recombinant AREG (B) and in the presence or absence of 100 ng/ml Gefitinib (C). Proliferation was defined as the percentage of cells that had undergone at least one division. Experiment performed in triplicate; bars represent means + SEM.

(D) Mouse FoxP3-GFP CD4+ T cells were cultured for 4 days together with CFSE-labeled CD45.1-expressing splenocytes at a ratio of 1:4 in the presence or absence of 100 ng/ml recombinant AREG. T cells were activated with different amounts of soluble anti-CD3, and CFSE dilution within the CD45.1-expressing CD8+ T cell population was analyzed by flow cytometry. Proliferation was defined as the percentage of cells that had undergone at least one division. Triangles, inhibition in the presence of AREG; squares, inhibition in the absence of AREG. Experiments performed in triplicate; points are means ± SEM.

For additional information, see Figure S3.

dose-dependent manner in both $Rag1^{-/-}$ and $Areg^{-/-}Rag1^{-/-}$ mice. However, transferred Treg cells were significantly less suppressive in $Areg^{-/-}Rag1^{-/-}$ than in $Rag1^{-/-}$ mice (Figure 4A). Because the total number of Treg cells as well as the frequency of Treg cells within the T cell population recovered from the mesenteric lymph nodes and spleen correlated more with the state of inflammation than with the genetic background of the animal (data not shown), we concluded that also in vivo AREG does not influence the proliferation or survival of transferred T cells but directly enhances the suppressive capacity of Treg cells.

To verify that the AREG-mediated effect was directly mediated via Treg cell-expressed EGFR, we crossed $Egfr^{flox/flox}$ mice onto a $Cd4$-cre background and transferred sorted Treg cells based on CD25 expression derived from WT and from $Egfr^{flox/flox} \times Cd4$-cre mice into $Rag1^{-/-}$ mice. Both CD25-expressing populations contained similar frequencies of FoxP3-expressing cells (Figure S4G); however, as shown in Figure 4B, EGFR gene-deficient Treg cells were significantly less capable of suppressing the development of colitis than Treg cells derived from WT mice. EGFR gene-deficient naive CD4+ T cells, however, were fully able to induce colitis upon transfer into $Rag1^{-/-}$ mice and Treg cells derived from WT mice could efficiently suppress their effector function (Figure 4C). These data show that EGFR-mediated signaling does not affect the functionality or the regulation of effector T cells, although at the same time Treg cells are directly dependent on AREG-induced signals for optimal functioning in vivo.

Amphiregulin Protects B16 Tumors against Tumor Immunization

EGFR-targeting treatment methodologies are well established in the clinic for the treatment of tumors. Specific side effects, such as skin rashes, have been observed after treatment that could suggest treatment-associated immune dysregulation. At the same time, has it been shown that Treg cells can constitute an

important escape mechanism by which tumors protect themselves against tumor-specific immune responses (Zou, 2006). To test the hypothesis that the EGFR-targeting treatments could affect Treg cell function, we performed tumor immunization experiments in the presence and absence of an EGFR-blocking antibody and Gefitinib, an EGFR-specific tyrosine kinase inhibitor (TKI). We used the B16 melanoma model, for which the pivotal role of Treg cells in the generation of a tumor-intrinsic immuno-suppressive environment is well established (Sutmuller et al., 2001). So can tumor immunization-induced rejection of transplanted B16-F10 tumors be achieved in WT C57BL/6 mice, if Treg cells are depleted prior to tumor transfer and immunization (Sutmuller et al., 2001). Moreover, B16-F10 tumors lack EGFR expression (Figure 5A). To test the relevance of AREG-EGFR interaction in Treg cell functioning in this tumor model, we immunized B16 transplanted mice with TRP2$_{180-188}$ tumor epitope-pulsed in vitro differentiated bone marrow-derived dendritic cells (BM-DCs) 5 and 7 days after tumor transplantation. Concomitant to immunization, mice were treated with EGFR-blocking nanobodies every second day or, as a control (Matsushita et al., 2008), once with a low dose of cyclophosphamide (Figure 5B). As described before (Sutmuller et al., 2001; Matsushita et al., 2008), immunization alone had no effect on tumor growth in C57BL/6 mice. Also, nanobody or cyclosphosphamide treatment each by itself exerted no substantial influence on tumor growth. The combination of immunization with nanobody treatment, however, significantly enhanced the efficacy of the peptide-pulsed BM-DC immunization (Figure 5B). A similar enhanced efficacy of peptide-pulsed BM-DC immunization was obtained after concomitant treatment with the EGFR-specific tyrosine kinase inhibitor Gefitinib (Figure 5C), although slightly less pronounced than observed by EGFR-blocking nanobody treatment. This slightly lower efficacy is explained most probably by the short serum half-life of Gefitinib of only approximately 6 hr, because of rapid excretion through the kidney.
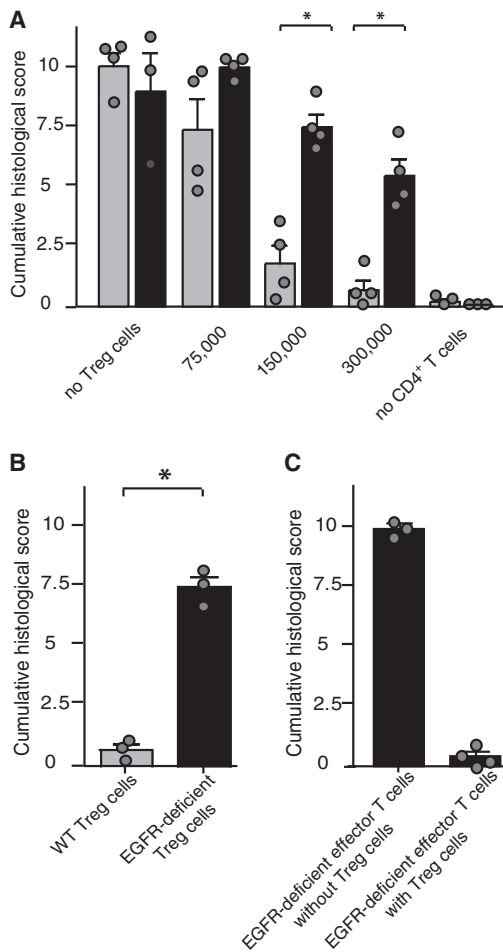
Figure 4. Amphiregulin Enhances Treg Cell Function In Vivo

(A) $Rag1^{-/-}$ (light bars) or $Areg^{-/-}Rag1^{-/-}$ (dark bars) mice received 400,000 flow cytometry-sorted naive CD4^{+} T cells together with increasing numbers of FoxP-GFP-expressing Treg cells.

(B) $Rag1^{-/-}$ mice received 400,000 naive CD4^{+} T cells together with 200,000 CD25^{+}CD4^{+} T cells derived from either WT (light bar) or $Egfr^{flox/flox} \times Cd4\text{-}cre$ (dark bar) mice.

(C) $Rag1^{-/-}$ mice received 400,000 naive CD4^{+} T cells derived from $Egfr^{flox/flox} \times Cd4\text{-}cre$ mice in the presence or absence of 200,000 CD25^{+}CD4^{+} T cells derived from WT mice.

Development of colitis was measured 6 weeks later by histological score. Bars represent means + SEM; results for individual mice are shown as circles. For additional information and representative H&E staining of tissue samples derived from the different mouse groups, see Figure S4.

Taken together, our data show that EGFR-targeted treatments can facilitate the rejection of a transplanted tumor that does not express the EGFR, when applied concomitant to CD8^{+} T cell-inducing antitumor immunization. These data indicate that EGFR-mediated signals are of critical importance for Treg cell-mediated establishment of a tumor-intrinsic immune-suppressive environment.

To establish that the observed effects of EGFR-blocking treatment indeed are AREG dependent and mediated via Treg cell-expressed EGFR, we transferred B16-F10 melanoma cells into WT C57BL/6, $Areg^{-/-}$, or $Egfr^{flox/flox} \times Cd4\text{-}cre$ mice and

immunized them with TRP2$_{180-188}$ tumor epitope-pulsed BM-DCs 5 and 7 days after tumor transplantation. B16 tumors grew with similar characteristics in the different mouse strains (Figures 5D and 5E) (although consistently somewhat faster in AREG-deficient than in WT mice, as shown in Figure 5D); however, unlike WT mice, $Areg^{-/-}$ (Figure 5F) and $Egfr^{flox/flox} \times Cd4\text{-}cre$ (Figure 5G) mice efficiently rejected transplanted B16-F10 tumors upon immunization. Because BM-DC immunization induced similar anti-TRP2$_{180-188}$-specific immune responses in the different mouse strains (data not shown) and we were not able to detect EGFR expression in CD8^{+} T cells (Figure 2B), we concluded that AREG-mediated enhancement of Treg cell function is of critical importance for the establishment of a tumor-intrinsic immune-suppressive environment in B16 tumors that prevents successful tumor rejection upon immunization in mice.

Mast Cell-Derived Amphiregulin Enhances Regulatory T Cell Function

Next we investigated the physiologically relevant source of AREG that enhances Treg cell function in vivo. Because Treg cells and mast cells have been shown to cooperate with each other (Lu et al., 2006; Hershko et al., 2011) and AREG has been shown to be the single most upregulated growth factor upon mast cell activation (Figure S5; Wang et al., 2005; Okumura et al., 2005), we tested whether mast cells could be a source of AREG in our models. To this end, we used mast cell-deficient $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ mice (Grimbaldeston et al., 2005), which were reconstituted with in vitro differentiated BM-derived mast cells (BM-MCs) prior to B16 transplantation and DC immunization. As shown in Figure 6A, $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ control mice that did not receive in vitro differentiated BM-MCs or mice that had been reconstituted with BM-MCs differentiated from BM from $Areg^{-/-}$ mice efficiently controlled tumor growth after peptide-specific immunization. In contrast, immunized mice reconstituted with BM-MCs differentiated from BM derived from WT C57BL/6 mice failed to control B16 growth. Because tumor growth was similar in all unimmunized $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ mouse groups (Figure 6B) and similar numbers of mast cells were detected in the peritumoral region surrounding blood vessels (Figure 6C) in all reconstituted $c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ mice, these data indicate that in the absence of AREG-expressing mast cells, Treg cell function was impaired.

Experiments performed in the T cell transfer-induced colitis model (Figures 6D and 6E) and the BM transfer-induced dermatitis model (Figure S1B) confirmed the critical role of mast cell-derived AREG in enhancing Treg cell function. Colitis scores in $c\text{-}kit^{w\text{-}sh/w\text{-}sh}RAG1^{-/-}$ mice that had been reconstituted with BM-MCs differentiated from BM of WT mice prior to transfer were 6 weeks after cotransfer of naive CD4^{+} T cells and Treg cells significantly lower than in mice that had not received BM-MCs or had been reconstituted with BM-MCs differentiated from BM of $Areg^{-/-}$ mice (Figure 6D). Transfer of naive CD4^{+} T cells only led to similar severe forms of colitis in all $c\text{-}kit^{w\text{-}sh/w\text{-}sh}RAG1^{-/-}$ mouse groups (Figure 6E), indicating that neither mast cells nor mast cell-derived AREG were necessary for the functioning or differentiation of colitogenic effector T cells. Also, cotransfer of BM-MCs differentiated from BM derived from WT C57BL/6 mice, but not of BM-MCs differentiated from BM derived from $Areg^{-/-}$ mice, prevented the
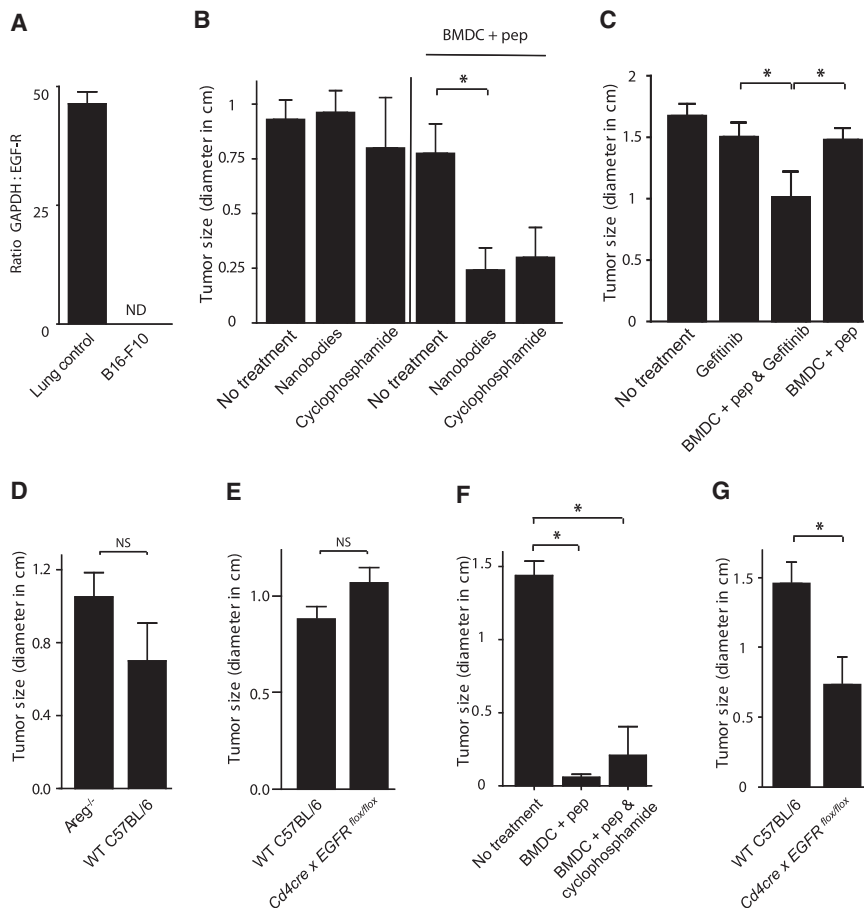
Figure 5. AREG Is of Critical Importance for the Efficient Suppression of Antitumor Immune Responses

(A) mRNA from B16-F10 tumor cells and control lung tissue was purified and quantitative RT-PCR was performed. Bar is mean of ratio GAPDH:EGFR expression + SEM. ND, nondetectable (no signal for EGFR).

(B and C) 1×10^4 B16-F10 cells were injected s.c. into the left flank of WT C57BL/6 mice. On days 5 and 7 after tumor cell transfer, mice were immunized with BM-DCs loaded with an immunogenic B16 melanoma epitope (TRP2$_{180-188}$) or left unimmunized. One mouse group received in addition 300 μg purified EGFR-blocking nanobodies (B) or 200 μg Gefitinib (C) every other day for 1 week starting from day 5.

(D–G) 1×10^4 B16-F10 cells were injected s.c. into WT C57BL/6 or $AREG^{-/-}$ (D and F) mice or $Egfr^{flox/flox} \times Cd4$-cre mice (E and G). On day 5 and 7 after tumor cell transfer, mice were immunized with BM-DCs loaded with an immunogenic B16 melanoma epitope (TRP2$_{180-188}$) (F and G) or left unimmunized (D and E). Tumor size was determined on days 21 until 23 after tumor transfer. n = 3–7 mice per group; bars represent mean + SEM.

development of dermatitis of WT C57BL/6 BM-chimeric mice that had received $Areg^{-/-}$ BM (Figure S1B).

These data obtained in mast cell-reconstituted mice indicate that leukocyte-, in this case mast cell-derived, AREG can be of crucial importance for efficient Treg cell functioning in vivo.

DISCUSSION

The pivotal role of Treg cells in immune regulation, i.e., in fine tuning protective immune responses and minimizing harmful pathology, has become widely appreciated. Despite this appreciation, many aspects of the highly complex process of how Treg cells are activated and, upon entry of inflamed tissues, are regulated, remain incompletely understood. Different factors and cell types have been shown to play a prominent role in this process, and a number of factors have been identified that influence the functionality of Treg cells at the site of inflammation. So far, mainly factors that diminish Treg cell function, such as IL-6 or TLR-mediated signals, have been described. Importantly, however, experiments have demonstrated that Treg cells gain optimal suppressive capacity only by migrating through the site of inflammation (Zhang et al., 2009). This finding suggests that at the site of inflammation also factors that enhance Treg cell function must be present. In line with this thought, we here demonstrate that AREG-induced and Treg cell-expressed EGFR-mediated signals are of pivotal importance for the optimal functioning of Treg cells in vivo. In particular, the observations that AREG is the single most upregulated growth factor upon activation of human mast cells (Wang et al., 2005) and that mast cell-derived AREG can be of critical importance for optimal Treg cell function shed a new light on the interaction between mast cells and Treg cells. Both Treg cells and mast cells rapidly infiltrate sites of inflammation and mutual regulation has repeatedly been described (reviewed in Mekori and Hershko, 2012). So far, however, mainly factors such as the cytokines IL-9 (Lu et al., 2006) and IL-2 (Hershko et al., 2011) have been described to be involved in this cross talk between mast cells and Treg cells. The main function of these cytokines, however, is to support survival and proliferation of mast cells (Townsend et al., 2000) and Treg cells (Burchill et al., 2007), respectively. Thus, interference with either of these factors has strong influences on the overall number of either type of cells. The finding presented here, that mast cell-derived AREG can directly enhance Treg cell function, adds a qualitative component to this interaction.

In the situation of developing tumors, both mast cells and Treg cells are known to accumulate in the peritumoral region (Ju et al., 2009) and we found that a particularly high percentage of Treg cells in the ectopic lymphoid structures at the periphery of tumors expressed the EGFR. Accumulation of mast cells around tumors has repeatedly been associated with poor prognosis for patients suffering from a number of tumors such as prostate cancer (Johansson et al., 2010), melanoma (Ribatti et al., 2003), breast cancer (Amini et al., 2007), or colon carcinoma (Lachter et al., 1995). Mast cells have been shown to support tumor growth in a number of different ways, for example, by enhancing the vascularization of the tumor, in particular in the
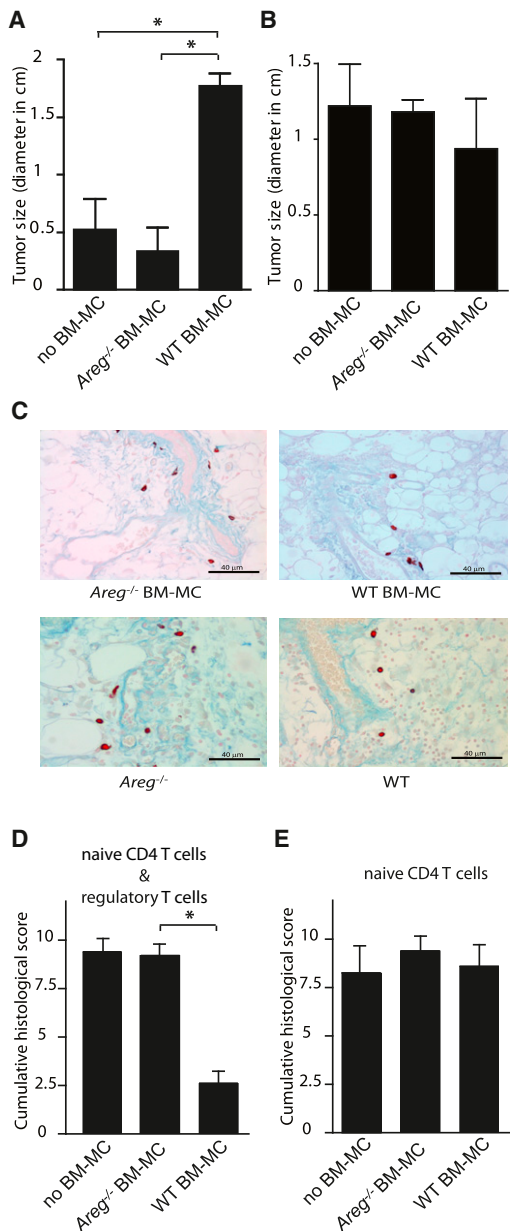
Figure 6. Mast Cell-Derived AREG Enhances Treg Cell Function In Vivo

(A and B) 1 × 10^4 B16-F10 cells were injected s.c. into *c-kit*^w-sh/w-sh mice reconstituted with BM-MCs differentiated from BM derived from WT C57BL/6 or *Areg*^−/− mice or were left unreconstituted. On day 5 and 7 after tumor cell transfer, mice were immunized with BM-DCs loaded with an immunogenic B16 melanoma epitope (TRP2_{180–188}) (A) or left unimmunized (B). Tumor size was determined 23 days after tumor transfer. n = 5–7 mice per group. Bars represent means + SEM.

(C) *c-kit*^w-sh/w-sh mice were reconstituted with either WT or *Areg*^−/− BM-MCs. Three weeks after reconstitution, 10,000 B16-F10 cells were injected. 21 days after tumor cell transfer, tumors were harvested and stained for mast cells via Csaba staining.

(D and E) *c-kit*^w-sh/w-sh*Rag1*^−/− mice were reconstituted with BM-MCs derived from either WT C57BL/6 or *Areg*^−/− mice or were left unreconstituted. 200,000 FoxP3/GFP CD4^+ T cells were cotransferred with 400,000 naive CD4^+ T cells and colitis scores were determined 6 weeks after transfer. n = 3–7 mice per group. Bars represent means + SEM.

For additional information, see Figure S6.

early phase of tumor development (Murdoch et al., 2008). Nevertheless, a contribution of mast cells to the tumor-intrinsic immune-suppressive environment is well established as well (Huang et al., 2008). Our data described here, that in particular mast cell-derived AREG substantially contributes to the immune-suppressive environment within tumors, suggest a molecular mechanism by which mast cells may support this Treg cell-mediated tumor-intrinsic immunosuppressive environment.

In line with this hypothesis, it is tempting to speculate that the immune-regulatory function of the EGFR may contribute to the clinical successes of EGFR-targeting medications. Tumor treatments often have multiple, sequential effects. Starting with the direct killing of a portion of the tumor cells, the thereby released tumor antigens can prime antitumor immune responses de novo or reactivate dormant, anergic responses (Apetoh et al., 2007). Our findings suggest that EGFR-targeting treatments may, in addition, also allow treatment-induced antitumor immune responses to be more effective, because the treatment may also interfere with the suppressive capacity of tumor-intrinsic Treg cells that otherwise could dampen the induced antitumor immune response.

Our findings may explain also why EGFR-targeting treatments in some tumors, such as colorectal carcinoma (Cunningham et al., 2004), function primarily as an adjuvant to concomitant chemotherapy (CT). CT induces cell death that results in release of tumor antigens that can prime or reactivate antitumor T cell responses and diminishes the overall tumor load temporarily and, thus, the therewith associated immune-suppressive environment (Zitvogel et al., 2008). More importantly, CT also induces a transient lymphopenia in which dormant and/or anergic tumor-specific T cell responses can be reactivated (Schietinger et al., 2012). Thus, in patients undergoing CT treatment, a similar situation is created as in our T cell transfer-induced colitis model in *Rag1*^−/− mice. Most interestingly, also in many other settings the enhancing effects of transient lymphopenia on the induction of autoimmune responses have been observed (Krupica et al., 2006; Guerau-de-Arellano et al., 2009) and in particular in such lymphopenic situations, optimal Treg cell function has been shown to be of pivotal importance for maintenance of immune tolerance and tissue homeostasis (Le Campion et al., 2009). Thus, it is easy to envision that blocking of the EGFR on Treg cells in combination with CT can enhance the efficacy of tumor treatments—which closely correlates with what is observed in the clinic (Cunningham et al., 2004). The exact mechanisms by which EGFR-targeted treatments mediate their objective clinical responses in cancer patients has remained poorly understood and further targeted research to determine the precise role of the immune system in the clinical successes of EGFR-targeted tumor treatments is needed. Nevertheless, our data showing a direct effect of the EGFR on Treg cell regulation may reveal one unexpected effect of such treatments. A much wider usage of EGFR-targeting treatments to enhance antitumor immune responses concomitant to other tumor treatments may therefore appear warranted.

Thus, taken together, we here reveal an EGFR-mediated mechanism that contributes to the regulation of local immune responses. Although factors that diminish Treg cell function at the site of inflammation have been known for many years, we

here describe a factor that enhances Treg cell function. Optimal immune regulation probably will follow from the balance between the two types of factors. It is easy to envision that either type of factor could have a distinct relevance at different time points of inflammation. During the acute phase, factors that diminish Treg cell function most probably prevail, but when the inflammation has cooled down, mast cell-derived AREG could play a more prominent role and thereby prevent excessive tissue damage and the development of chronic inflammations.

EXPERIMENTAL PROCEDURES

Mice

$c\text{-}kit^{w\text{-}sh/w\text{-}sh}$ and FoxP3-GFP transgenic mice were purchased from Jackson Laboratory, *CD4-cre* mice were purchased from Taconics, and *Egfr*$^{flox/flox}$ mice (Natarajan et al., 2007) were provided by M. Sibilia (University of Vienna). *Areg*$^{-/-}$, which had been backcrossed for at least 14 times onto a C57BL/6 background, B6.SJL (CD45.1), and *Rag1*$^{-/-}$ mice were bred in house under specific-pathogen-free conditions. Mice were used between 7 and 17 weeks of age. All animal experiments were approved by the Committee on Animal Experiments of the University of Utrecht.

Nanobody Isolation

Because EGFR-blocking antibodies target the human EGFR only and do not cross-react with the murine EGFR, we first screened antibodies derived from llama immunoglobulin libraries for their capacity to interact with mouse EGFR. Besides conventional immunoglobulins, llamas express single-domain, heavy chain antibodies devoid of the light chain, known as nanobodies. We isolated three nanobody-producing clones that targeted a stretch of the EGFR that is conserved between mouse and human (Figure S6). To enhance the nanobody half-life in the serum of treated mice, a bivalent, bispecific nanobody construct was prepared by cloning an anti-mouse albumin nanobody at the C terminus of RR359, separated by a 15(G4S) linker; resulting in a serum half-life of this bi-head nanobody of approximately 48 hr (data not shown). Bivalent RR359 was expressed in *S. cerevisiae* shake flask cultures and purified via C-His tag affinity with 1 ml HisTrap column. For FACS staining, the nanobody was dialyzed to PBS with 5 ml HiTrap Desalting column (both GE Healthcare) and biotinylated with biotin (amido hexanoic acid 3-sulpho-N-hydroxy succinimide ester, Roche); staining was performed in the presence of Fc-block and/or unspecific, unlabeled nanobodies.

B16 Melanoma Vaccination

10,000 B16-F10 melanoma cells were transferred subcutaneously into the lower left flank of mice. Mice were then either immunized with TRP2$_{180-188}$ peptide-loaded BM-DCs on day 5 and 7 after transfer or left untreated. On day 6 after tumor transfer, mice were i.p. injected every other day either with 10 mg/kg bodyweight Gefitinib or with 300 μg purified EGFR-blocking nanobodies. Assuming a half-life of about 48 hr, the level of nanobodies per mouse was kept above 100 μg for 1 week. A control group received once on day 5 after transfer an i.p. injection of 50 mg/kg body weight cyclophosphamide dissolved in 100 μl PBS to inactivate Treg cells. 21 days after injection the tumor size was determined. Up to a diameter of approximately 0.8 cm, tumors normally grew perfectly round. At bigger sizes, space restrainments formed more oval-shaped tumors; of these the average of width versus length was calculated.

Induced Colitis Mouse Model

Rag1$^{-/-}$ mice were injected with 4×10^5 CD4$^+$CD45RB^{hi} cells to induce colitis. If not stated differently in the figure legend, 2×10^5 Treg cells isolated from Foxp3-GFP mice were cotransferred and 6 weeks later the mice were sacrificed and colons scored by two independent experts in a blinded fashion according to Berg et al. (1996). Scoring was as follows, in brief: grade 0, no infiltration of mononuclear cells; grade 1, few foci of mononuclear cells, only slight depletion of goblet cells; grade 2, many foci of mononuclear cells, infiltration in the lamina propria, but not yet in the submucosa; and diminished numbers of goblet cells; grade 3, strong infiltration, also in the submucosa; epithelial hyperplasia; and number of goblet cells strongly diminished; grade 4, transmural infiltration of mononucleated cells; strong epithelial hyperplasia; and goblet cell depletion. Overall histological score per mouse is the sum of individual scores for the different segments of the colon (c. ascendens, c. transversum, c. descendens).

Differentiation and Reconstitution of Bone Marrow-Derived Mast Cells

Bone marrow-derived mast cells (BM-MCs) were differentiated in vitro from bone marrow cells that were cultured for 3 weeks in the presence of pokeweed mitogen-stimulated spleen cell-conditioned medium as a source for IL-3. Nonadherent cells were passed once a week into new medium and purity of BM-MC population was determined by flow cytometry. Cultures contained a uniform cell population to more than 94% positive for c-Kit and FcεRIα. Mast cell-deficient *c-kit*$^{w\text{-}sh/w\text{-}sh}$ mice with an age of at least 8 weeks were i.v. injected and received 5×10^6 cultured BM-MCs 3 weeks prior to start of the experiments. Mast cell reconstitution of the inflamed area was determined by Csaba staining and toluidine blue.

In Vitro Suppression Assay

Human

CD4$^+$CD25^{hi}CD127^{lo} T cells isolated from human PBMCs were cocultured with PBMCs labeled with 2 μM CFSE in anti-CD3 (clone OKT3)-coated 96-well plates. Cells were cultured for 4 days in RPMI medium supplemented with 10% FCS, penicillin and streptomycin, and 2-mercaptoethanol, in the presence or absence of 100 ng/ml recombinant Amphiregulin, purified from the supernatant of COS7 cells that had been transiently transfected with AREG-expressing vectors or purchased from R&D Systems. Proliferation of CD4$^+$ and CD8$^+$ cells was determined by measuring CFSE dilution with the FACS CANTO (BD Biosciences). Proliferation was defined as the percentage of cells that have undergone at least one division.

Mouse

FACS-sorted Treg cells were added to CFSE-labeled CD45.1-expressing splenocytes at different ratios. Cells were cultured in IMDM supplemented with 10% FCS, glutamax, penicillin and streptomycin, and 2-mercaptoethanol for 4 days, in the presence or absence of 100 ng/ml recombinant AREG. T cells were activated with different amounts of soluble anti-CD3 (clone 145-2C11; BD PharMingen). To determine suppression of proliferation, CFSE dilution within the CD45.1-expressing T cell populations was analyzed by FACS. Proliferation was defined as the percentage of cells that have undergone at least one division.

Statistics

We performed data analysis with statistical software (Prizm 4.0, GraphPad Software). Comparisons between two groups were performed with two-tailed Mann-Whitney test. p values <0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.09.023.

REFERENCES

Altan-Bonnet, G., and Germain, R.N. (2005). Modeling T cell antigen discrimination based on feedback control of digital ERK responses. PLoS Biol. *3*, e356.

Amini, R.M., Aaltonen, K., Nevanlinna, H., Carvalho, R., Salonen, L., Heikkilä, P., and Blomqvist, C. (2007). Mast cells and eosinophils in invasive breast carcinoma. BMC Cancer *7*, 165.

Apetoh, L., Ghiringhelli, F., Tesniere, A., Obeid, M., Ortiz, C., Criollo, A., Mignot, G., Maiuri, M.C., Ullrich, E., Saulnier, P., et al. (2007). Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat. Med. *13*, 1050–1059.

Avraham, R., and Yarden, Y. (2011). Feedback regulation of EGFR signalling: decision making by early and delayed loops. Nat. Rev. Mol. Cell Biol. *12*, 104–117.

Berg, D.J., Davidson, N., Kühn, R., Müller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W., and Rennick, D. (1996). Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. J. Clin. Invest. *98*, 1010–1020.

Bonner, J.A., Harari, P.M., Giralt, J., Azarnia, N., Shin, D.M., Cohen, R.B., Jones, C.U., Sur, R., Raben, D., Jassem, J., et al. (2006). Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N. Engl. J. Med. *354*, 567–578.

Burchill, M.A., Yang, J., Vang, K.B., and Farrar, M.A. (2007). Interleukin-2 receptor signaling in regulatory T cell development and homeostasis. Immunol. Lett. *114*, 1–8.

Chan, G., Nogalski, M.T., and Yurochko, A.D. (2009). Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility. Proc. Natl. Acad. Sci. USA *106*, 22369–22374.

Chen, F., Liu, Z., Wu, W., Rozo, C., Bowdridge, S., Millman, A., Van Rooijen, N., Urban, J.F., Jr., Wynn, T.A., and Gause, W.C. (2012). An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. Nat. Med. *18*, 260–266.

Chung, K.Y., Shia, J., Kemeny, N.E., Shah, M., Schwartz, G.K., Tse, A., Hamilton, A., Pan, D., Schrag, D., Schwartz, L., et al. (2005). Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. J. Clin. Oncol. *23*, 1803–1810.

Cobbold, S.P., Adams, E., Graca, L., Daley, S., Yates, S., Paterson, A., Robertson, N.J., Nolan, K.F., Fairchild, P.J., and Waldmann, H. (2006). Immune privilege induced by regulatory T cells in transplantation tolerance. Immunol. Rev. *213*, 239–255.

Cunningham, D., Humblet, Y., Siena, S., Khayat, D., Bleiberg, H., Santoro, A., Bets, D., Mueser, M., Harstrick, A., Verslype, C., et al. (2004). Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N. Engl. J. Med. *351*, 337–345.

Ferris, R.L., Jaffee, E.M., and Ferrone, S. (2010). Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoescape. J. Clin. Oncol. *28*, 4390–4399.

Garrido, G., Lorenzano, P., Sánchez, B., Beausoleil, I., Alonso, D.F., Pérez, R., and Fernández, L.E. (2007). T cells are crucial for the anti-metastatic effect of anti-epidermal growth factor receptor antibodies. Cancer Immunol. Immunother. *56*, 1701–1710.

Grimbaldeston, M.A., Chen, C.C., Piliponsky, A.M., Tsai, M., Tam, S.Y., and Galli, S.J. (2005). Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. Am. J. Pathol. *167*, 835–848.

Guerau-de-Arellano, M., Martinic, M., Benoist, C., and Mathis, D. (2009). Neonatal tolerance revisited: a perinatal window for Aire control of autoimmunity. J. Exp. Med. *206*, 1245–1252.

Hershko, A.Y., Suzuki, R., Charles, N., Alvarez-Errico, D., Sargent, J.L., Laurence, A., and Rivera, J. (2011). Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. Immunity *35*, 562–571.

Huang, B., Lei, Z., Zhang, G.M., Li, D., Song, C., Li, B., Liu, Y., Yuan, Y., Unkeless, J., Xiong, H., and Feng, Z.H. (2008). SCF-mediated mast cell infiltration and activation exacerbate the inflammation and immunosuppression in tumor microenvironment. Blood *112*, 1269–1279.

Ishikawa, N., Daigo, Y., Takano, A., Taniwaki, M., Kato, T., Hayama, S., Murakami, H., Takeshima, Y., Inai, K., Nishimura, H., et al. (2005). Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. Cancer Res. *65*, 9176–9184.

Jacobs, B., De Roock, W., Piessevaux, H., Van Oirbeek, R., Biesmans, B., De Schutter, J., Fieuws, S., Vandesompele, J., Peeters, M., Van Laethem, J.L., et al. (2009). Amphiregulin and epiregulin mRNA expression in primary tumors predicts outcome in metastatic colorectal cancer treated with cetuximab. J. Clin. Oncol. *27*, 5068–5074.

Johansson, A., Rudolfsson, S., Hammarsten, P., Halin, S., Pietras, K., Jones, J., Stattin, P., Egevad, L., Granfors, T., Wikström, P., and Bergh, A. (2010). Mast cells are novel independent prognostic markers in prostate cancer and represent a target for therapy. Am. J. Pathol. *177*, 1031–1041.

Ju, M.J., Qiu, S.J., Gao, Q., Fan, J., Cai, M.Y., Li, Y.W., and Tang, Z.Y. (2009). Combination of peritumoral mast cells and T-regulatory cells predicts prognosis of hepatocellular carcinoma. Cancer Sci. *100*, 1267–1274.

Khambata-Ford, S., Garrett, C.R., Meropol, N.J., Basik, M., Harbison, C.T., Wu, S., Wong, T.W., Huang, X., Takimoto, C.H., Godwin, A.K., et al. (2007). Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. J. Clin. Oncol. *25*, 3230–3237.

Krupica, T., Jr., Fry, T.J., and Mackall, C.L. (2006). Autoimmunity during lymphopenia: a two-hit model. Clin. Immunol. *120*, 121–128.

Lachter, J., Stein, M., Lichtig, C., Eidelman, S., and Munichor, M. (1995). Mast cells in colorectal neoplasias and premalignant disorders. Dis. Colon Rectum *38*, 290–293.

Le Campion, A., Gagnerault, M.C., Auffray, C., Bécourt, C., Poitrasson-Rivière, M., Lallemand, E., Bienvenu, B., Martin, B., Lepault, F., and Lucas, B. (2009). Lymphopenia-induced spontaneous T-cell proliferation as a cofactor for autoimmune disease development. Blood *114*, 1784–1793.

Lu, L.F., Lind, E.F., Gondek, D.C., Bennett, K.A., Gleeson, M.W., Pino-Lagos, K., Scott, Z.A., Coyle, A.J., Reed, J.L., Van Snick, J., et al. (2006). Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature *442*, 997–1002.

Mahtouk, K., Hose, D., Rème, T., De Vos, J., Jourdan, M., Moreaux, J., Fiol, G., Raab, M., Jourdan, E., Grau, V., et al. (2005). Expression of EGF-family receptors and amphiregulin in multiple myeloma. Amphiregulin is a growth factor for myeloma cells. Oncogene *24*, 3512–3524.

Matsumoto, K., Fukuda, S., Nakamura, Y., and Saito, H. (2009). Amphiregulin production by human eosinophils. Int. Arch. Allergy Immunol. *149*(*Suppl 1*), 39–44.

Matsushita, N., Pilon-Thomas, S.A., Martin, L.M., and Riker, A.I. (2008). Comparative methodologies of regulatory T cell depletion in a murine melanoma model. J. Immunol. Methods *333*, 167–179.

Mekori, Y.A., and Hershko, A.Y. (2012). T cell-mediated modulation of mast cell function: heterotypic adhesion-induced stimulatory or inhibitory effects. Front. Immunol. *3*, 6.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. (2009). Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity *30*, 899–911.

Montagut, C., Dalmases, A., Bellosillo, B., Crespo, M., Pairet, S., Iglesias, M., Salido, M., Gallen, M., Marsters, S., Tsai, S.P., et al. (2012). Identification of a mutation in the extracellular domain of the Epidermal Growth Factor Receptor conferring cetuximab resistance in colorectal cancer. Nat. Med. *18*, 221–223.

Murdoch, C., Muthana, M., Coffelt, S.B., and Lewis, C.E. (2008). The role of myeloid cells in the promotion of tumour angiogenesis. Nat. Rev. Cancer *8*, 618–631.

Natarajan, A., Wagner, B., and Sibilia, M. (2007). The EGF receptor is required for efficient liver regeneration. Proc. Natl. Acad. Sci. USA *104*, 17081–17086.

Okumura, S., Sagara, H., Fukuda, T., Saito, H., and Okayama, Y. (2005). FcepsilonRI-mediated amphiregulin production by human mast cells increases mucin gene expression in epithelial cells. J. Allergy Clin. Immunol. *115*, 272–279.

Powrie, F., Leach, M.W., Mauze, S., Menon, S., Caddle, L.B., and Coffman, R.L. (1994). Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity *1*, 553–562.

Qi, Y., Operario, D.J., Oberholzer, C.M., Kobie, J.J., Looney, R.J., Georas, S.N., and Mosmann, T.R. (2010). Human basophils express amphiregulin in response to T cell-derived IL-3. J. Allergy Clin. Immunol. *126*, 1260–1266.e4.

Ribatti, D., Ennas, M.G., Vacca, A., Ferreli, F., Nico, B., Orru, S., and Sirigu, P. (2003). Tumor vascularity and tryptase-positive mast cells correlate with a poor prognosis in melanoma. Eur. J. Clin. Invest. *33*, 420–425.

Schietinger, A., Delrow, J.J., Basom, R.S., Blattman, J.N., and Greenberg, P.D. (2012). Rescued tolerant CD8 T cells are preprogrammed to reestablish the tolerant state. Science *335*, 723–727.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. Cell *103*, 211–225.

Stern, K.A., Place, T.L., and Lill, N.L. (2008). EGF and amphiregulin differentially regulate Cbl recruitment to endosomes and EGF receptor fate. Biochem. J. *410*, 585–594.

Sutmuller, R.P., van Duivenvoorde, L.M., van Elsas, A., Schumacher, T.N., Wildenberg, M.E., Allison, J.P., Toes, R.E., Offringa, R., and Melief, C.J. (2001). Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J. Exp. Med. *194*, 823–832.

Tinhofer, I., Klinghammer, K., Weichert, W., Knödler, M., Stenzinger, A., Gauler, T., Budach, V., and Keilholz, U. (2011). Expression of amphiregulin and EGFRvIII affect outcome of patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment. Clin. Cancer Res. *17*, 5197–5204.

Townsend, J.M., Fallon, G.P., Matthews, J.D., Smith, P., Jolin, E.H., and McKenzie, N.A. (2000). IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. Immunity *13*, 573–583.

Tsang, J.Y., Camara, N.O., Eren, E., Schneider, H., Rudd, C., Lombardi, G., and Lechler, R. (2006). Altered proximal T cell receptor (TCR) signaling in human CD4+CD25+ regulatory T cells. J. Leukoc. Biol. *80*, 145–151.

Wang, S.W., Oh, C.K., Cho, S.H., Hu, G., Martin, R., Demissie-Sanders, S., Li, K., Moyle, M., and Yao, Z. (2005). Amphiregulin expression in human mast cells and its effect on the primary human lung fibroblasts. J. Allergy Clin. Immunol. *115*, 287–294.

Zaiss, D.M., Yang, L., Shah, P.R., Kobie, J.J., Urban, J.F., and Mosmann, T.R. (2006). Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. Science *314*, 1746.

Zhang, N., Schröppel, B., Lal, G., Jakubzick, C., Mao, X., Chen, D., Yin, N., Jessberger, R., Ochando, J.C., Ding, Y., and Bromberg, J.S. (2009). Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity *30*, 458–469.

Zitvogel, L., Apetoh, L., Ghiringhelli, F., and Kroemer, G. (2008). Immunological aspects of cancer chemotherapy. Nat. Rev. Immunol. *8*, 59–73.

Zou, W. (2006). Regulatory T cells, tumour immunity and immunotherapy. Nat. Rev. Immunol. *6*, 295–307.