

Letter to the Editor

Regulatory T-cell-intrinsic amphiregulin is dispensable for suppressive function

To the Editor:

Amphiregulin is 1 of 7 structurally and functionally related growth factors that bind the epidermal growth factor receptor (EGFR); it is present in membrane-bound, intracellular and secreted forms. Interest in a role for amphiregulin in the immune system has been growing: various cells from both the innate and adaptive arms of the immune system express the ligand, including basophils,¹ several subsets of human T cells, and murine T_H2 cells.² Populations of tissue-specific murine CD4⁺Foxp3⁺ regulatory T (Treg) cells in the colon and striated muscle are enriched in amphiregulin during inflammation; indeed, amphiregulin plays a role in repair functions of muscle Treg cells.³ Treg cells transferred to amphiregulin^{-/-} RAG^{-/-} mice show suboptimal ability to prevent colitis in an adoptive transfer model, a phenotype that is rescued by amphiregulin supplementation.⁴ To further understand the potential role of amphiregulin in Treg-cell function and its role in inflammation, the abundance and functional significance of amphiregulin

expressed by Treg cells from the peripheral lymphoid tissues of mice was investigated (see the [Methods](#) section and [Table E1](#) in this article's Online Repository at www.jacionline.org). Transcriptomic analysis revealed differential expression of amphiregulin between conventional T cells (Tcons) and Treg cells ([Fig 1, A and B](#)): amphiregulin mRNA was greater than 3-fold more abundant in Treg cells than in Tcons, confirmed by quantitative RT-PCR ([Fig 1, A, B, and C](#)). Heparin-binding epidermal growth factor-like growth factor (HB-EGF) also showed greater expression by Treg cells than by Tcons, as did EGFR itself ([Fig 1, A-C](#)); the remaining ligands fell below the limits of detection (data not shown).

Stimulation of the T-cell receptor (TCR) in human CD3⁺ T cells, CD4⁺ T cells,⁵ and murine T_H2, but not T_H1,⁶ cells, induces amphiregulin expression. In human CD4⁺ T cells, TCR engagement also augments HB-EGF expression.⁵ To determine whether a similar response occurs in murine Treg cells and Tcons (both naive and memory), we examined the kinetics of expression following TCR stimulation. A spike in amphiregulin expression was observed in all 3 populations at 3 hours ([Fig 1, Di](#)). Highest expression of amphiregulin mRNA at every time point was apparent in the Treg cells, followed by the mTcons and then nTcons. A significant difference between the regulatory and naive T cells was observed at every time point ([Fig 1, Di](#)).

There was no significant difference in HB-EGF expression in Treg cells over the time course, remaining higher than both Tcon

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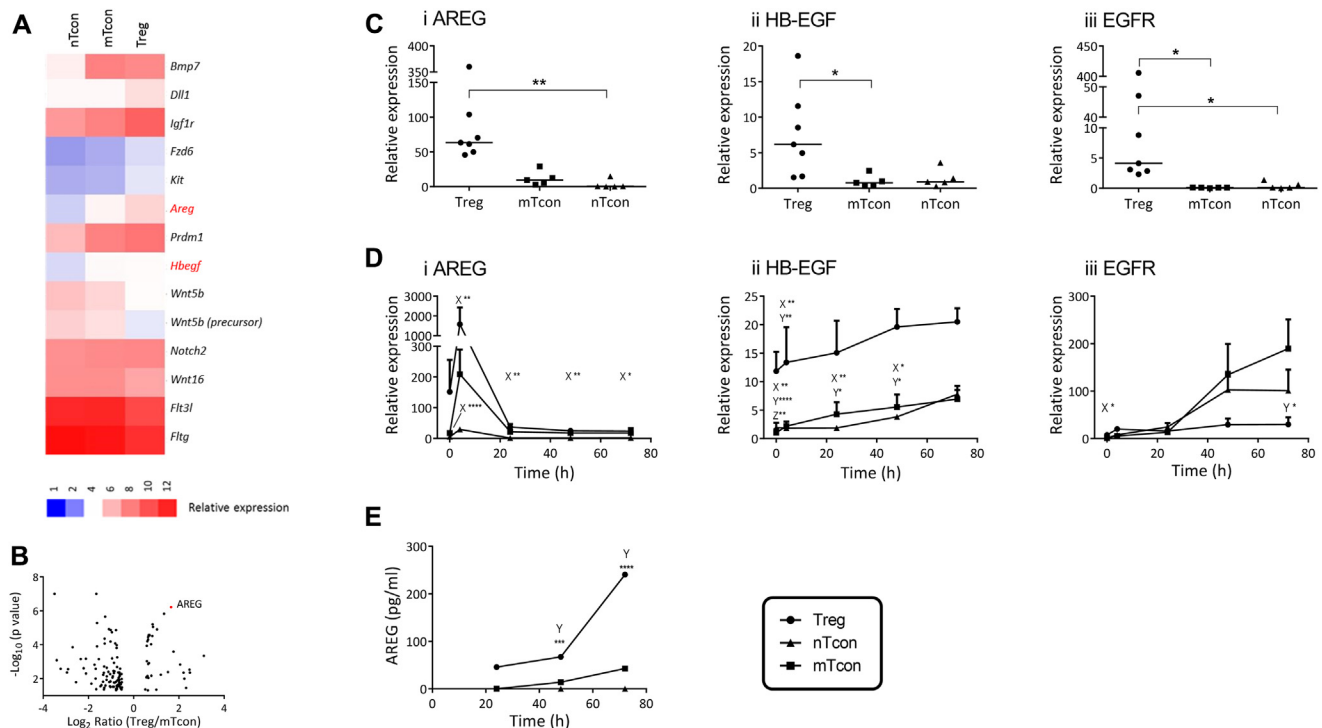


FIG 1. Treg cells show an amphiregulin^{high} phenotype. Peripheral Treg cells and memory (m) and naive (n) Tcons selected from naive WT mice revealed greater amphiregulin and HB-EGF transcript abundance by microarray analysis (**A**: heat map; **B**: volcano plot) and quantitative RT-PCR assays (**C**; $n \geq 5$), both *ex vivo* and following polyclonal stimulation (**D**, $n = 3$; **E**, $n \geq 2$). AREG, Amphiregulin. Key: Statistical significance between X = Treg cells/nTcons, Y = Treg cells/mTcons, and Z = nTcons/mTcons (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$).

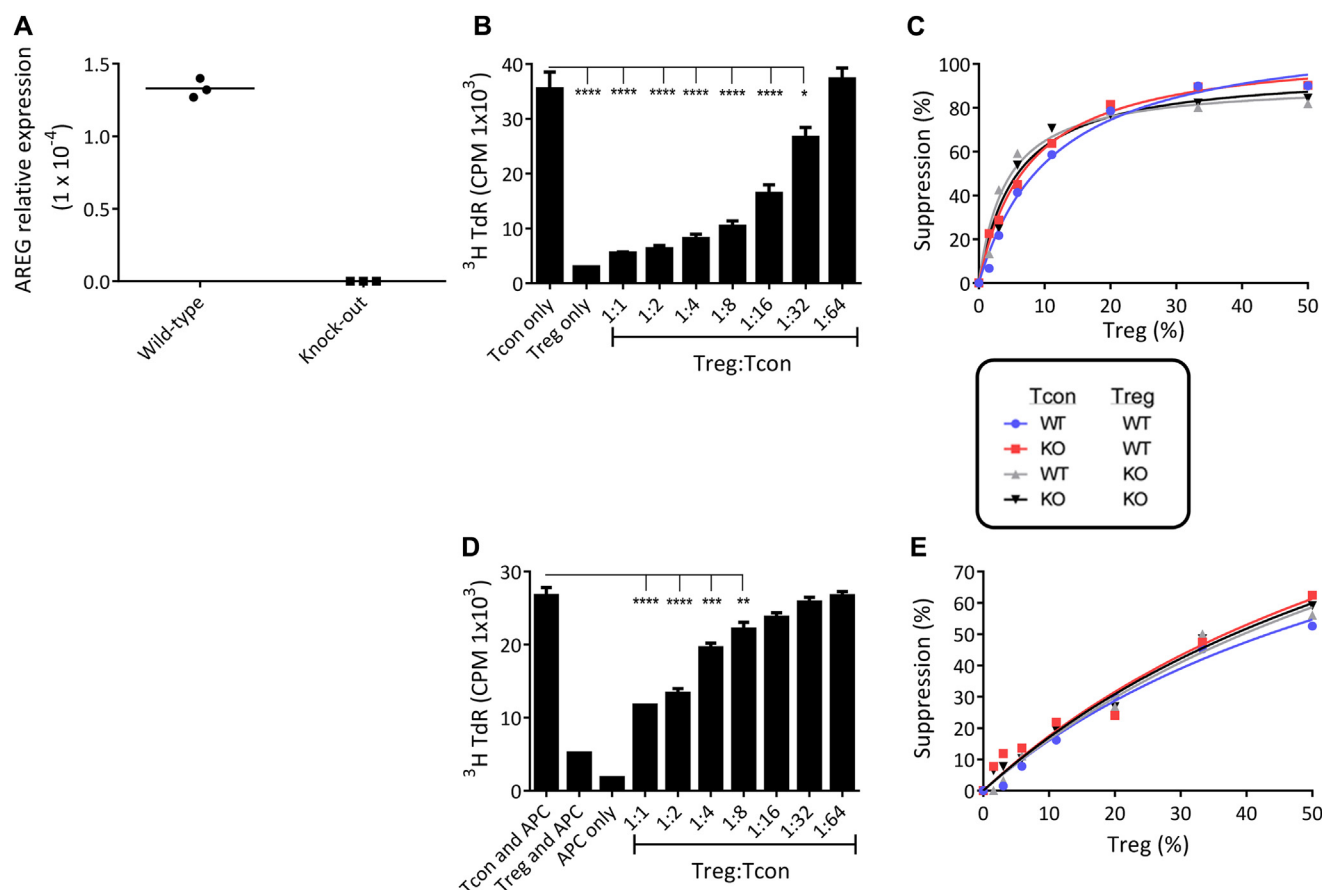


FIG 2. Treg-cell-intrinsic amphiregulin is not required for suppressive function *in vitro*. **A**, Confirmation of genotype by quantitative RT-PCR. Cocultured Tcons and Treg cells were stimulated with (**B** and **C**) anti-CD3/CD28-coated Dynabeads or (**D** and **E**) soluble anti-CD3 with WT antigen-presenting cells (APCs). **B** and **D**, Counts per minute (CPM) in 1 representative experiment (* $P = .011$, ** $P = .0065$, *** $P = .0002$, **** $P < .0001$); **C** and **E**, proportional suppression of CPM. **A**, $n = 1$; **B** and **C**, $n = 3$; **D** and **E**, $n = 1$. AREG, Amphiregulin.

populations at each point except 72 hours (Fig 1, Dii). In both subsets of Tcons, EGFR mRNA expression displayed a sustained increase from the first to the final time point (Fig 1, Diii). To determine whether the differential expression of the mRNA encoding amphiregulin and HB-EGF reflected differences in protein abundance, secreted amphiregulin and HB-EGF were quantified in culture supernatants. Amphiregulin secreted from nTcons remained below the assay detection limit of 7.8 pg/mL. The concentration of amphiregulin in the Treg-cell supernatants exceeded that of mTcons from 48 hours onward (Fig 1, E). Concentrations of HB-EGF were below the lower limit of detection in all supernatant samples (data not shown).

To establish whether the higher amphiregulin expression observed in Treg cells reflected a role in their suppressive function, bead-based assays using syngeneic and cross-over cocultures of Treg cells and Tcons from wild-type (WT) and amphiregulin knock-out (KO) mice were performed, following confirmation of their genotype (Fig 2, A). An inhibitory effect was apparent at all Treg-cell:Tcon ratios, with no difference between WT and KO cells; in particular, KO:KO cocultures, with neither an intrinsic nor an extrinsic source of amphiregulin in the cultures, showed potent suppression (Fig 2, B and C). Moreover, recombinant amphiregulin added to cultures of T cells *in vitro*

impacted neither their proliferation nor their apoptosis, showing no evidence of an inhibitory role (data not shown). The suppression assays were repeated using WT accessory cells and soluble anti-CD3 mAb to activate cocultures of WT and KO Treg cells and Tcons, to establish whether Treg-cell suppression *via* antigen-presenting cells requires an intrinsic source of amphiregulin. Once again, there was no difference in suppression between any of the coculture permutations.

A regulatory role for amphiregulin is not intuitive, as the ligand has a number of proinflammatory associations including its induction of the cytokines IL-1 α and IL-1 β , which positively feed back to promote amphiregulin secretion.⁷ However, other studies have found a role for the ligand in the function of various subsets of murine Treg cells.² Augmentation of human Treg-cell-mediated suppression of CD4⁺ Tcons *in vitro* in response to recombinant amphiregulin was observed by Zaiss et al.⁴ In the same study, enhanced activity of murine Treg cells was also apparent in response to recombinant amphiregulin, but CD8⁺ T cells were used as the responder population in these experiments, with no information on the inhibitory effect on murine CD4⁺ Tcons. The ligand also enhances Treg-cell suppression of the antiviral effects of CD8⁺ T cells *in vivo*.⁸ Amphiregulin may therefore augment Treg-cells'

suppressive activity in a CD8⁺ T-cell-specific manner, but whether amphiregulin itself represents a mechanism of suppression by Treg cells in certain cellular contexts remains unknown.

Our data demonstrate that despite being more abundantly expressed by Treg cells than by Tcons, Treg-cell-intrinsic amphiregulin is not required for suppressive function *in vitro* because functional redundancy was demonstrated in 2 well-established *in vitro* assays of suppression, suggesting that amphiregulin cannot be considered a core regulatory mechanism of these cells. Rather, we speculate that amphiregulin is involved in the tissue-reparative effects of Treg cells, in a manner that is independent of regulatory function. This viewpoint is supported by recently published work that complements our study^{2,9} and extends our current insight into the complex, multifaceted roles of amphiregulin in health and disease.

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Methods

Mice

AREG^{tm1Dle/Mmnc} mice on a C57BL/6J x 129S1/SvJ background were obtained from the Mutant Mouse Regional Resource Centre (MMRRC; University Of North Carolina, Chapel Hill, USA). The homozygous null mutant mice were bred onto a C57BL/6J background (obtained from Charles River Laboratories, UK) in the GlaxoSmithKline (GSK) Laboratory Animal Sciences department (Stevenage, UK), confirming zygosity by PCR. Following multiple generations of backcrossing to C57BL/6J, the genetic background of the strain was confirmed by single-nucleotide polymorphism analysis using the MAX-BAX[®] platform (Charles River Laboratories) to be greater than 98% C57BL/6J. To generate animals for study, heterozygous AREG^{tm1Dle/Mmnc} breeding pairs were mated to generate homozygous AREG knock-out and wild-type litter-mate controls. All animal procedures were reviewed and approved by the GSK Animal Care and Use Committee, and were performed in AAALAC accredited facilities in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Laboratory Animals. Mice were genotyped, weaned, and separated based on sex at three weeks of age, then multiply-housed in open-rack caging at an ambient temperature 19–21°C and humidity 45–55% maintained on a 06:00h:20:00h light–dark cycle, with free access to maintenance diet (LabDiet[®] custom 5LFL) and animal-grade drinking water. GSK animal facilities are maintained at Specific Pathogen Free status, with routine health monitoring carried out to FELASA recommendations.

Cell isolation

Following Schedule 1 euthanasia by exposure to CO₂ gas in a rising concentration and confirmation of death by cervical dislocation, lymph nodes and spleens were harvested from mice, macerated and washed with RPMI-1640. Erythrocytes were lysed using ammonium chloride-potassium bicarbonate buffer. Fluorescence-activated cell sorting (FACS[™]) was employed to isolate Treg (CD4⁺CD25⁺CD45RB^{low}), naïve (n) Tcon (CD4⁺CD25⁻CD45RB^{high}) and memory (m) Tcon (CD4⁺CD25⁻CD45RB^{low}) populations. Cells were stained with antibodies against CD4, CD45RB and CD25 (all eBioscience, Hatfield, UK), before FACS[™] on a MoFlo[™] cell sorter (Beckman Coulter, High Wycombe, UK).

Microarray

An RNeasy mini kit (QIAGEN, Manchester, UK) was used for total RNA extraction, following the manufacturer's protocol. A microarray assay was performed using the Glyco-gene Chip v4, containing 1,127 probe sets targeting glycosylation-related transcripts including genes encoding proteins that are either heavily glycosylated or involved in glycosylation, as well as 119 probe sets targeting reference ('housekeeping') transcripts (see Gene Lists). Robust Multichip Average (RMA) software was used to convert intensity to expression values¹ and Cluster software was used for hierarchical clustering. Analysis of variance performed by BRB Array Tools was used to identify genes on the arrays showing significant differences at or below a 20% false discovery rate, focusing on changes greater than 1.4-fold. Heat maps were generated with JavaTreeView. All transcriptomic data have been uploaded to the National Institutes of Health Gene Expression Omnibus database, accession number GSE31921 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31921>).

Quantitative reverse transcriptase (RT)-PCR

Gene Expression Assays (Primer Design, Southampton, UK) containing double-dye probes with non-complementary primers specific to the target transcripts were used with Precision OneStep[™] qRT-PCR MasterMix (Primer Design). Reactions with >38 cycle quantification (C_q) were discounted from analysis. Samples were run in triplicate.

For each transcript standard curves were generated, plotting the C_q values against the dilution factor and using the slope to calculate amplification efficiency. These values were used to calculate the abundance of transcript in mRNA from cells of interest, relative to a control population of unselected CD4⁺ T cells at time 0, using Pfaffl's model².

Six candidate reference genes were screened for stability across the populations of interest using the geNorm system. *Actb* and *Gapdh* were selected.

Cell culture

Cells were cultured in round bottom 96-well plates in RPMI-1640 supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 10mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic and 10% v/v fetal calf serum (complete medium). All culture conditions, including those of suppression assays, were set up in triplicate.

ELISA

Secreted AREG was measured in culture supernatants by means of an ELISA DuoSet kit for murine AREG (R&D Systems, Minnesota, USA); complete medium at time 0 contained no detectable AREG (data not shown). Owing to a lack of a commercially available murine heparin-binding EGF-like factor (HB-EGF) ELISA, a human HB-EGF ELISA kit (R&D Systems) was used, generating a standard curve with murine recombinant HB-EGF (ProSpec, East Brunswick, New Jersey, USA). The assay detection limit for murine HB-EGF was 125pg/ml. All samples were run in triplicate.

Suppression assays

Tregs and nTcons were co-cultured in complete medium. Proliferation was induced by means of anti-CD3/CD28 mAb-coated Dynabeads[®] (Invitrogen) or mitomycin C-treated (25µg/mL) splenocytes in the presence of 250ng/ml anti-CD3 mAb for three days. To measure proliferation, cells were pulsed with tritiated thymidine (³H-TdR) for 18 hours before measurement of ³H-TdR incorporation with a cell harvester and liquid scintillation counter (Wallac Trilux, Perkin Elmer, UK).

Statistical tests

Statistical significance of transcript expression (Fig 1) was determined by the Kruskal-Wallis test followed by the Dunn's multiple comparisons test; a linear mixed model followed by Holm's Bonferroni correction was used to obtain post-hoc *p* value cut-off points. Horizontal bars in the figures represent median values, while error bars represent standard errors of the mean. Statistical significance of suppression (Fig 2) was determined using one-way ANOVA followed by the Holm Sidak's multiple comparison test. Significance was assumed when *p*<0.05.

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