

Amphiregulin, a T_H2 Cytokine Enhancing Resistance to Nematodes

Dietmar M. Zaiss,¹ Li Yang,¹ Pranav R. Shah,¹ James J. Kobie,¹ Joseph F. Urban,² Tim R. Mosmann^{1*}

Despite improved sanitation, intestinal nematode infections remain a major health threat to humans. Protection against nematodes is mainly mediated by type 2-biased immune responses (1), characterized by T helper 2 (T_H2) lymphocytes and other cells secreting a set of cytokines, including interleukin 4 (IL-4), IL-5, IL-10, and IL-13 (2). Increased shedding of the epithelium of the caecum of the intestine is also crucially important for clearing the intestinal nematode parasite *Trichuris muris* (3). Members of the epidermal growth factor (EGF) family can induce proliferation of the gut epithelium (4). GeneChip (Affymetrix, Santa Clara, CA,) analysis of genes induced in different CD4 T cell subsets revealed that in vitro activation through the T cell antigen receptor induced preferential expression of the EGF family member amphiregulin in T_H2 cells, compared with the absence of amphiregulin expression in naïve, T_Hpp, or T_H1 cells (Fig. 1A). Selective expression in T_H2 cells was confirmed by a GeneChip analysis of T cell subsets from a second mouse strain (5) and by a Northern blot and reverse transcription polymerase chain reaction (RT-PCR) analysis (fig. S1).

To test whether amphiregulin was also expressed during a typical type 2 response in vivo, we infected C57BL/6 mice with *T. muris*, and 14 days after infection we analyzed cDNA from mesenteric lymph nodes (MLN) by real-time PCR. Expression of the T_H1 cytokine interferon- γ was reduced, whereas expression of amphiregulin was increased, in parallel with the typical T_H2 cytokines IL-4 and IL-13 (Fig. 1B). Amphiregulin mRNA was also detected by real-time PCR [normalized threshold cycle difference (Δ Ct) = 11.5

compared to CD3 ϵ] in the caecal tissue of infected amphiregulin-deficient mice (6) that had received CD4 T cells from a *Trichuris*-infected wild-type mouse but was undetectable (Δ Ct > 15.9 compared to CD3 ϵ) in nontransferred mice, confirming amphiregulin expression by T cells in the caecal tissue.

The physiological relevance of amphiregulin to parasite infection was tested by comparing amphiregulin-deficient and wild-type C57BL/6 mice after infection with *T. muris*. In these experiments, similar numbers of larvae were detected after 10 days (366 compared to 363), and both wild-type and amphiregulin-deficient mice cleared the parasite by day 19. However, worm clearance at day 14 was significantly delayed in amphiregulin-deficient mice (Fig. 1C). The CD4 T cell-dependent increase in the progression of proliferating epithelial cells up

the gut villi, induced by *Trichuris* infection, was also diminished in amphiregulin-deficient mice (fig. S2). However, the infection induced similar IL-4, IL-5, and IL-13 responses in amphiregulin-deficient and wild-type mice (fig. S3).

Clearance of *Trichuris* is independent of mast cells, γ δ T cells, eosinophils, and natural killer T cells (1). However, it remained possible that nonlymphoid gut cells might produce amphiregulin that could enhance pathogen clearance. To test whether lymphoid cell-derived amphiregulin was sufficient to accelerate parasite clearance, we infected bone marrow chimeras between amphiregulin-deficient and wild-type mice with *Trichuris* and measured the worm burdens 14 days later. Amphiregulin-expressing bone marrow cells significantly restored parasite clearance in amphiregulin-deficient mice ($P = 0.021$, one-tailed Mann Whitney test, Fig. 1D). Thus, although other pathways (possibly IL-13-dependent) can ultimately expel worms, amphiregulin secreted by hematopoietic (probably T_H2) cells significantly enhances the expulsion of this intestinal parasite.

The expression of amphiregulin by the T_H2 subset of T cells provides a newly recognized link between the type 2 adaptive immune response and the proliferation of gut epithelial cells that aids helminth parasite removal. This response adds to other type 2 effector mechanisms, including edema and smooth muscle contractility (8), to provide a concerted attack on the parasites. The coexpression of amphiregulin with the allergy-inducing T_H2 cytokine pattern also raises the intriguing possibility that T_H2 cells contribute to both the tissue hypertrophy and allergic mechanisms that cause asthma.

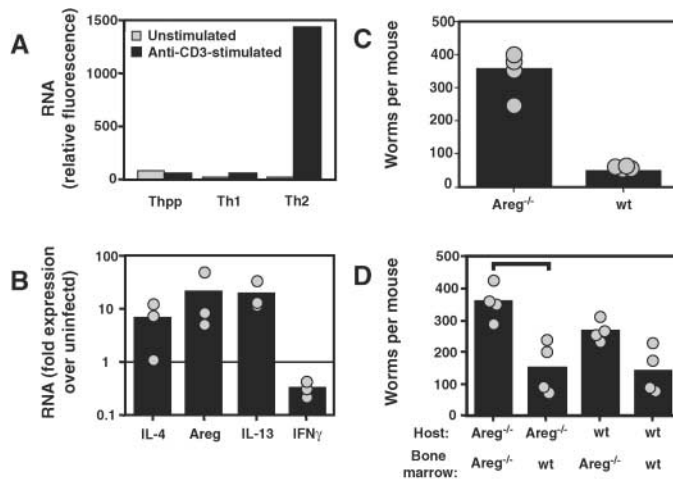


Fig. 1. Amphiregulin (Areg) is expressed by activated T_H2 cells and required for rapid nematode expulsion. (A) BALB/c T_Hpp, T_H1, and T_H2 cell lines were produced as described previously (5), and gene expression was measured by using Affymetrix GeneChips before and 9 hours after activation by antibodies specific for CD3 and CD28 (5, 7). (B) RNA isolated from MLN of day 14 *Trichuris*-infected C57BL/6 mice was analyzed by real-time PCR. Expression quantities were normalized to CD3 ϵ expression and compared with uninfected mice (baseline of Areg expression being Δ Ct = 12.8 compared to CD3 ϵ). (C) Female wild-type (wt) or amphiregulin-deficient mice were infected with *T. muris*, and worms in the caecum and upper colon were counted on day 14 by a blinded operator (6). (D) Irradiated (10 gray) wild-type and amphiregulin-deficient mice were reconstituted with either wild-type or amphiregulin-deficient T cell-depleted bone marrow cells and after 30 days infected with *T. muris*. Worms in the gut were counted blind on day 14.

References and Notes

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7. Materials and Methods are available on Science Online.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/314/5806/1746/DC1
Materials and Methods
Figs. S1 to S3

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Supporting Online Material for

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This PDF file includes:

Materials and Methods
Figs. S1 to S3

Material & Methods:

Analysis of gene expression:

RNA derived from different tissues or cell lines was purified by incubation with TRIzol (Invitrogen) according to the manufacturer's instructions, reverse transcribed using random hexamers and either analyzed by real-time PCR using TaqMan gene-specific probes (Applied BioSystems), or by RT-PCR or Northern blot analysis. Amounts of RNA was quantified by absorbance at 260nm and equal loading was confirmed by ethidium bromide staining of 5S and 28S rRNA. Blotted RNA or PCR products were hybridized with a ³²P labeled Amphiregulin-specific cDNA probe at 42°C in 50% formamide.

Worm Infection:

Wild-type C57BL/6 mice, or Amphiregulin-deficient (tenth backcross to C57BL/6) mice, were first familiarized to chocolate milk and then infected with *Trichuris muris* by offering 400 eggs in 0.2 ml chocolate milk to single mice after overnight water deprivation. Mice that did not readily drink the offered chocolate milk were not included in the studies. Worms in the caecum and upper colon were counted at different time points after infection by a blinded operator under a dissecting microscope after a 2 hrs incubation at 37°C in PBS containing 10µM EDTA, and vigorous vortexing.

BrdU labeling and analysis of epithelial cell movement:

Mice were injected i.p. with 0.1ml PBS containing 10mg BrdU (Sigma Aldrich). At different time points after injection, tissues were harvested and fixed in 10% formaldehyde (Sigma Aldrich). The following day, the fixative was replaced by 70% ethanol. Fixed tissues were embedded, cut and lightly stained with H&E. BrdU-labeled cells were visualized using BrdU specific antiserum (DAKO) according to the manufacturer's instructions.

Supplemental Figure S1:

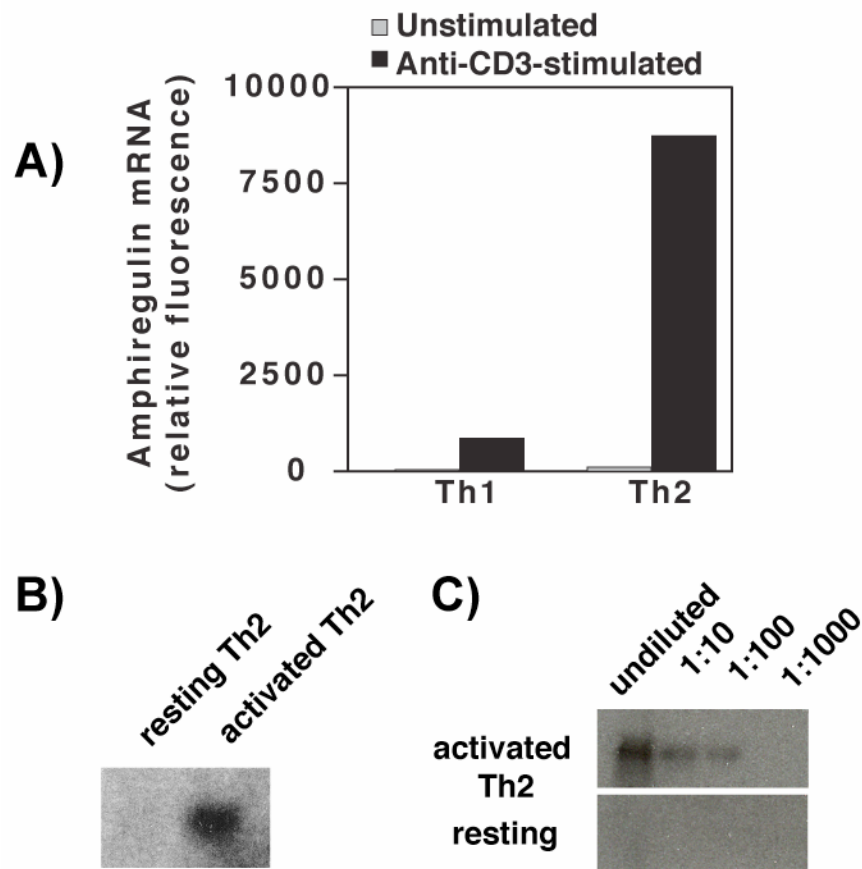
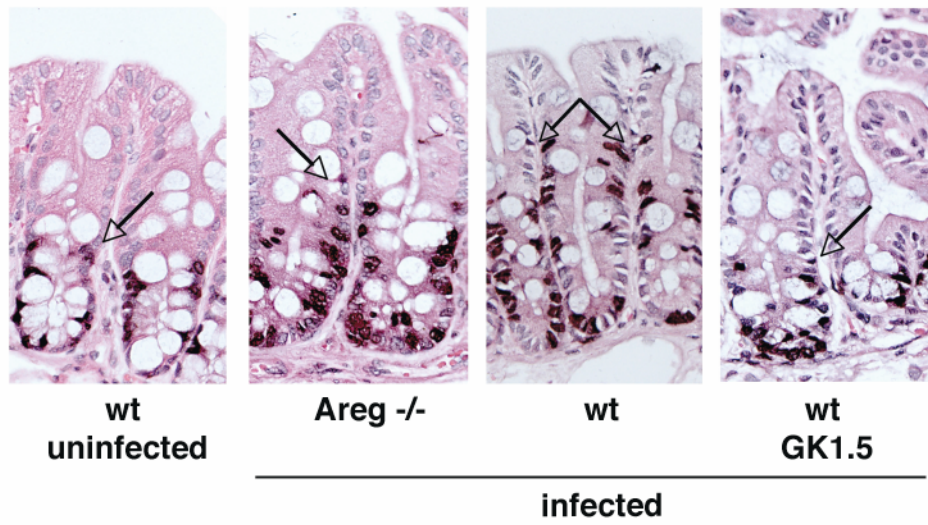


Fig. S1: Preferential expression of Amphiregulin in activated Th2 cells.

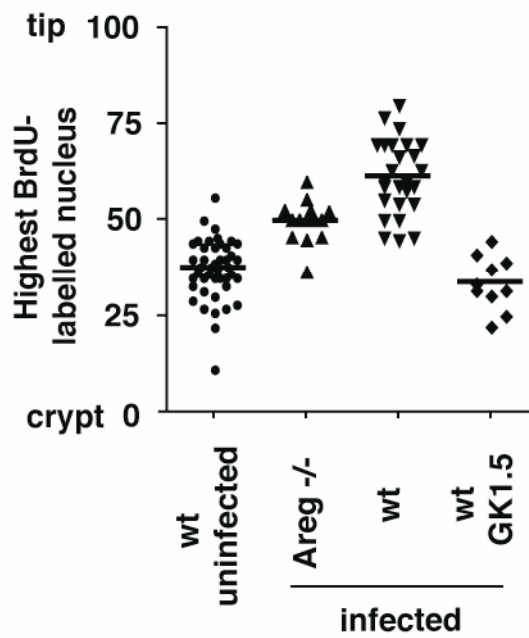
Resting, C57BL/6-derived long term Th1 and Th2 cell cultures were activated by plate bound anti-CD3/CD28 antibodies or were left untreated. After 9 hrs, RNA was isolated and Amphiregulin gene expression was quantified by Affymetrix GeneChip analysis (A), Northern Blot (B) or RT-PCR (C). Equal amounts of RNA derived from resting and activated Th2 cell cultures were used (B & C) and PCR products were blotted onto a membrane. To verify the specificity of the PCR products (C), membranes were hybridized with the same radioactive Amphiregulin-specific probe used for the Northern blot in (B).

Supplementary Figure S2:

A)



B)



C)

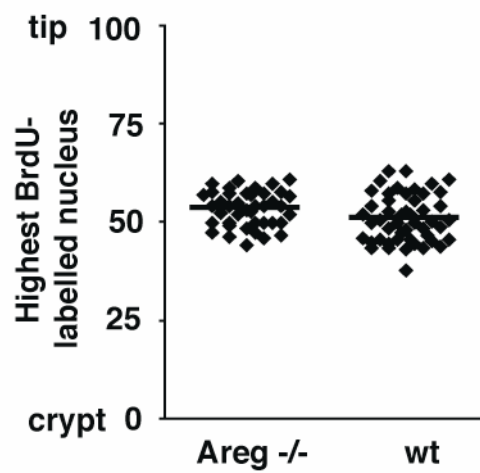


Fig. S2: Diminished epithelial cell movement in the caecum of infected Amphiregulin-/- mice.

C57BL/6 wt or Amphiregulin deficient mice were infected with *Trichuris* (A & B) or left uninfected (C). Five days after infection one group of wt mice (C) was depleted of CD4 T cells by *in vivo* treatment with GK1.5 antibody. At day 10 after infection (A & B), mice were pulsed with 10mg BrdU, and after a nine-hour overnight (A & B) or 24 hrs (C) chase, caecal tissues were harvested, fixed, embedded, cut and stained lightly with H&E. BrdU labeled cells were visualized using BrdU-specific antiserum. A). The migration of the highest BrdU-labeled cells, as a percentage of total villus length (B & C), was measured by a blinded observer in sections of villi that were straight and similarly sized. Oblique sections were not scored. Two mice/group (B).

Supplemental Figure S3:

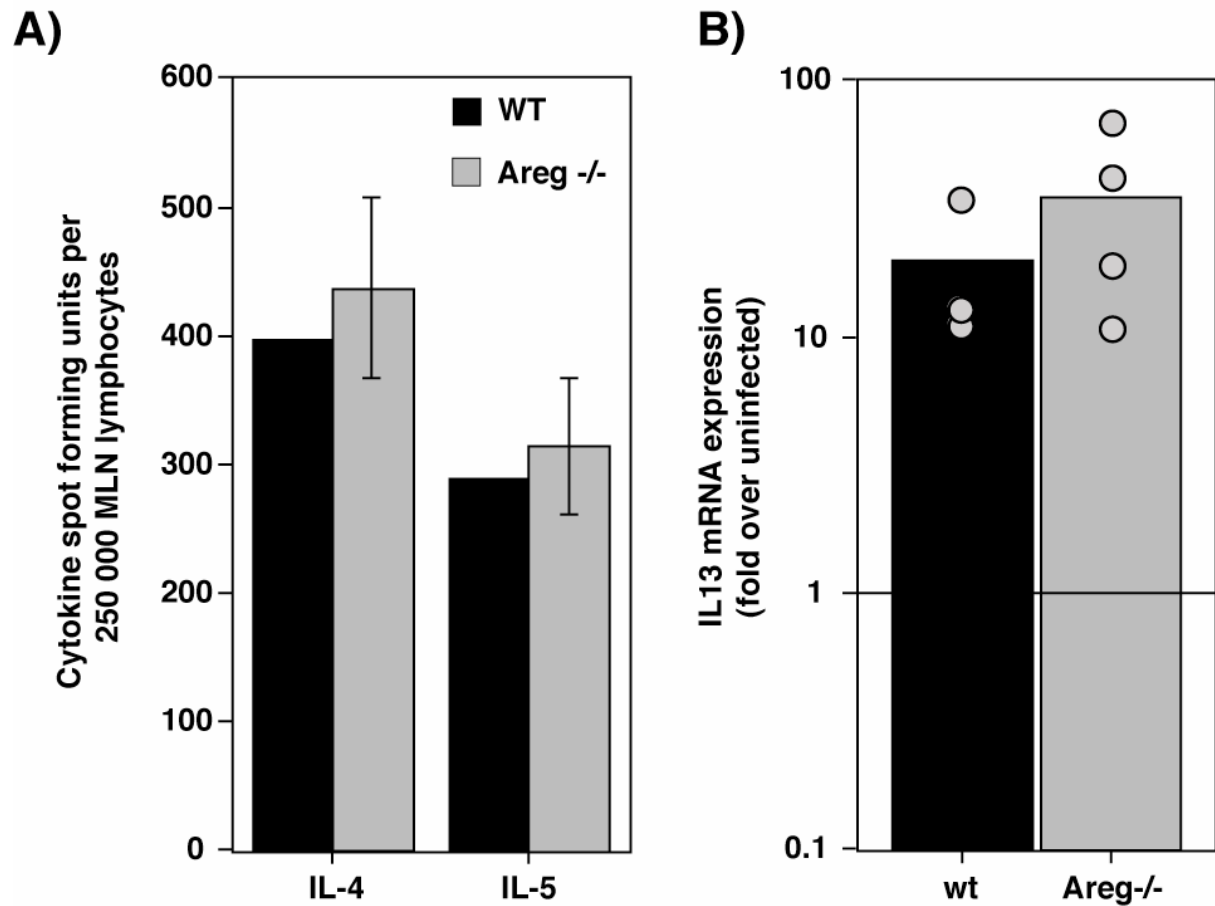


Fig. S3: Amphiregulin-deficient and C57BL/6 mice have similar Th2 responses.

C57BL/6 mice were infected with *Trichuris muris* for 14 days, and cytokine production by mesenteric lymph node cells in response to membrane-bound anti-CD3/anti-CD28 activation was measured by IL-4 and IL-5 Elispot assays (A), or by real-time PCR with IL13 specific TaqMan probes (B).