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HB-EGF Synthesized by CD4 T Cells Modulates Allergic Airway Eosinophilia by Regulating IL-5 Synthesis

Soroor Farahnak, Leora Simon, Toby K. McGovern, Michael Chen, Niusa Khazaei, and James G. Martin

CD4 T cells express the epidermal growth factor (EGF) receptor ligand, heparin-binding EGF (HB-EGF), with no defined immuno-pathophysiological function. Therefore, we wished to elucidate the function of HB-EGF synthesized by CD4 T cells in the context of allergic pulmonary inflammation and the asthma surrogate, airway hyperresponsiveness, in a murine acute model of asthma. In this study, we show how knocking out HB-EGF expression in CD4 T cells in vivo attenuates IL-5 synthesis in the lung that is accompanied by diminished eosinophilic inflammation and airway hyperresponsiveness. HB-EGF coimmunoprecipitates with the transcriptional repressor B cell lymphoma 6 (Bcl-6) in CD4 T cells. Knocking out HB-EGF in CD4 T cells resulted in increased Bcl-6 binding to the IL-5 gene and decreased IL-5 mRNA expression. Thus, these findings suggest an immunoregulatory function for intrinsic HB-EGF expressed by CD4 T cells in T_H2 inflammation and airway dysfunction by modulating IL-5 expression via binding to and inhibiting the repressive function of Bcl-6. *The Journal of Immunology*, 2019, 203: 000–000.

CD4 T cells play crucial roles in inflammatory diseases by differentiating into various subsets, each acquiring particular characteristics in the adaptive responses by secreting various patterns of cytokines, chemokines, and growth factors. Typically, $\alpha\beta$ TCR-expressing CD4 T cells are subdivided into T_H1 , T_H2 , T_H17 , follicular helper cells, and regulatory T cells, but more recently, the classification has been further expanded to include additional subtypes, including T_H3 , T_H9 , T_H23 , and T_H25 (1). In addition to the secretion of proinflammatory cytokines, T cells express growth factors, which may be anticipated to affect the physiology of the tissues. For example, CD4 T cells produce heparin-binding epidermal growth factor (EGF) (HB-EGF) and basic fibroblast growth factor 2 in humans (2). T_H2 cells synthesize amphiregulin, which contributes to intestinal epithelial proliferation in a murine model of helminth infection, facilitating the clearance of parasites (3).

HB-EGF signals through ErbB1 and ErbB4 directly and indirectly via ErbB2 and ErbB3 (4). HB-EGF is produced as a

transmembrane preformed protein that is cleaved at the extracellular domain by matrix metalloproteinases (MMPs) and members of the ADAM family of sheddases, resulting in the formation of soluble HB-EGF (sHB-EGF) and cytoplasmic HB-EGF (cHB-EGF). Upon ectodomain shedding, the cytoplasmic domain of pro-HB-EGF binds to and inhibits the function of gene transcriptional repressors B cell lymphoma 6 (Bcl-6) and promyelocytic leukemia zinc finger (5–7). However, HB-EGF interaction with Bcl-6 in immune cells has not been documented.

A transcriptional repressor, Bcl-6 is a sequence-specific multifunctional regulator of lymphocyte differentiation and immune responses for both T and B cells. Bcl-6 is a master regulator of follicular helper T cell differentiation as well as the expression of T_H2 cytokine genes (8). Bcl-6 has been shown to have a consensus binding site for IL-4 and IL-5 genes (9, 10). Bcl-6-deficient mice develop spontaneous eosinophilic inflammation in the lung, and in vitro activated CD4 T cells from Bcl-6-deficient mice have exaggerated the mRNA expression for IL-4, IL-5, and IL-13 (11). A T_H1 -polarizing milieu induces higher levels of Bcl-6 protein expression by CD4 T cells compared with T_H2 -skewing conditions at the early stages of polarization (12). The mechanisms controlling the regulation of Bcl-6 in CD4 T cells is unclear.

Asthma is characterized by chronic inflammation, airway hyperresponsiveness (AHR), and tissue remodeling (13). The allergic phenotype is predominantly associated with the presence of T_H2 cells. T_H2 cytokines and chemokines such as, IL-3, IL-4, IL-5, IL-13, GM-CSF, eotaxin, and RANTES have a pivotal role in eosinophilic inflammation in asthma (14). IL-5 is crucial for the development of eosinophils from bone marrow progenitors as well as promoting their migration, activation, and survival (15). Increased IL-5 mRNA expression in the bronchoalveolar lavage (BAL) fluid correlates with eosinophilic inflammation in asthmatic patients, whereas neutralization of IL-5 has been effective in reducing asthma exacerbations (16, 17). Eosinophils synthesize a variety of bioactive molecules, such as lipid mediators, IL-13, major basic protein, and eosinophil peroxidase, that may contribute to asthma pathogenesis and AHR (18).

The aim of this study was to investigate the immunological and pathophysiological properties of HB-EGF synthesized

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; Bcl-6, B cell lymphoma 6; cHB-EGF, cytoplasmic HB-EGF; ChIP, chromatin immunoprecipitation; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF; 4-OHT, 4-hydroxy tamoxifen; MMP, matrix metalloproteinase; PAS, Periodic acid-Schiff; PSA, penicillin-streptomycin-amphotericin; qPCR, quantitative PCR; sHB-EGF, soluble HB-EGF.

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by CD4 T cells. In the current study, we found that inducing acute asthma in mice with HB-EGF-deficient CD4 T cells, HB-EGF^{lox/lox}CD4CreER^{T2}, reduces AHR as well as IL-5 and eosinophilia in the lung. Our results further suggest that decreased IL-5 expression by HB-EGF-deficient lung resident T_H2 cells may be attributed to a diminished interaction of HB-EGF and Bcl-6, resulting in increased suppressive function of Bcl-6 by repressing IL-5 gene expression. Besides, in humans, there are a higher frequency of IL-5 and HB-EGF expression by peripheral T_H2 cells from asthmatic patients compared with nonasthmatics.

Materials and Methods

Animals

CD4CreER^{T2} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). HB-EGF lox knock-in mice were purchased from the RIKEN Institute (Wako, Japan). Bcl-6^{lox/lox}CD4cre and DO.11.10 mice were kindly provided by Dr. Irah King and Dr. Elizabeth Fixman (McGill University, Montreal, Canada), respectively. Male BALB/c and C57BL/6J mice, 8–10 wk of age, were purchased from Charles River Laboratories. Animals were bred in a conventional animal care facility at the McGill University Health Centre Research Institute. Protocols and procedures were approved by an institutional animal care committee and were in conformity with the standards of the Canadian Council of Animal Care.

Abs and chemicals

Anti-mouse CD16/32; FITC-, PerCP-, and allophycocyanin-conjugated anti-mouse CD4; V500-conjugated anti-human CD4; PE- and PerCP-conjugated anti-mouse CD11c; Brilliant Violet 510-conjugated anti-mouse CD11b; allophycocyanin-conjugated anti-mouse IL-5; and Brilliant Violet 421-conjugated donkey anti-rabbit IgG were purchased from BioLegend. eFluor 710-conjugated anti-mouse Siglec-F was purchased from Thermo Fisher Scientific. Anti-CD3, anti-CD28, and anti-IL-4 Abs were purchased from Becton Dickinson. Rabbit anti-HB-EGF polyclonal Ab was purchased from Bioss Antibodies. OVA, BSA, PMA (19), ionomycin, GM6001, tamoxifen-free base, 4-hydroxy tamoxifen (4-OHT), and peanut oil were purchased from Sigma-Aldrich. Abs against C-terminal domain of HB-EGF and an anti-Bcl-6 Ab were purchased from Santa Cruz Biotechnology. Rabbit and mouse IgG were obtained from Cell Signaling Technology.

Allergic sensitization and challenge

Naive BALB/c, C57BL/6, and Bcl-6^{lox/lox}CD4cre were sensitized with OVA (100 µg) and aluminum hydroxide (1 mg) (Thermo Fisher Scientific) in 100 µl of PBS (Invitrogen) by i.p. injection on days 1 and 7. One week following the second sensitization, under light isoflurane anesthesia, mice were either challenged on three consecutive days with OVA (50 µg) via intranasal instillation or their spleens were collected without challenge. Control mice were sensitized i.p. with OVA and challenged with PBS. Forty-eight hours following the third challenge, mice were euthanized, and lungs were harvested. HB-EGF^{lox/lox}CD4CreER^{T2} and C57BL/6 mice were sensitized with OVA on day 1 and 7. On day 13, mice were treated with tamoxifen-free base (Sigma-Aldrich) administered in peanut oil and 10% ethanol (5 mg/animal) by gavage for five consecutive days. Vehicle was administered to control mice. Five days after the last tamoxifen dosage, animals were challenged with OVA intranasally for three consecutive days. All experiments were conducted 48 h following the last challenge.

Tissue collection and cell culture

Spleens from naive DO.11.10 mice, naive and OVA-sensitized BALB/c mice, and HB-EGF^{lox/lox}CD4CreER^{T2} mice were harvested. Suspensions of splenocytes were prepared by mechanical disruption of the spleen, followed by passaging through 40-µm cell strainers. RBCs were lysed with ammonium chloride-potassium lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Cells were cultured in 24-well tissue culture plates in a concentration of 5×10^6 cells/ml in RPMI 1640 supplemented with FBS (10%) and penicillin–streptomycin–amphotericin (PSA) (Life Technologies) and incubated at 37°C with 5% CO₂. DO.11.10 CD4 T cells were stimulated by culturing splenocytes with OVA (200 µg/ml) for 3 d. To inhibiting IL-4 signaling pathway, anti-IL-4 Ab (10 µg/ml) was administered, and cells were cultured for 7 d. Every 3 d, half of the medium was replaced with fresh medium and anti-IL-4 Ab. Splenocytes harvested from OVA-sensitized BALB/c mice were incubated with OVA (200 µg/ml) in vitro for 4, 6, and 8 d. CD4 T cells were purified by MACS and positive

selection, following the manufacturer's instructions (Miltenyi Biotec). As an alternative mechanism of CD4 T cell stimulation, CD4 T cells were isolated from naive BALB/c and HB-EGF^{lox/lox}CD4CreER^{T2} splenocytes by MACS, cultured in anti-CD3 Ab (3 µg/ml) precoated plates, and treated with anti-CD28 Ab (1 µg/ml) for 3 d. To knock out HB-EGF, 18 h following stimulation, 4-OHT (1.5 µg/ml) prepared in methanol was added to the culture medium of HB-EGF^{lox/lox}CD4CreER^{T2} CD4 T cells. Control cells were treated with vehicle. Cells were harvested after 48 h.

Lungs from OVA-sensitized and challenged animals were digested in collagenase (*Clostridium histolyticum*, type IA, 150 collagen digestion units/ml; Sigma-Aldrich) for 1 h at 37°C. Cells were passed through 40-µm cell strainers, and RBCs were lysed with ammonium chloride-potassium lysis buffer. Cells were resuspended in RPMI 1640 and supplemented with FBS (10%) and PSA.

Human sample preparation

Healthy and asthmatic subjects were recruited under protocol approved by the Ethics Review Board of the McGill University Health Centre. The demographic information on asthmatic subjects is provided in Supplemental Table I. Blood was collected following informed consent. PBMCs were collected using Ficoll-Paque PLUS (GE Healthcare). CD4 T cells were purified using MACS and positive selection, and stimulated with anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) in RPMI 1640 supplemented with 10% FBS and 1% PSA for 72 h.

Intracellular cytokine assessment by flow cytometry

To assess intracellular cytokines, cells were stimulated with PMA (75 ng/ml) and ionomycin (1 µM) in the presence of GolgiPlug for 4 h. Cells were stained with viability dye eFluor780. Following blocking with anti-mouse CD16/32, cells were stained for surface markers, CD4, CD11b, Siglec-F, and CD11c in FACS buffer (3% FBS in PBS). After fixation and permeabilization using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences), cells were stained for intracellular markers IL-5 and HB-EGF. Stained cells were analyzed by LSRFortessa cell analyzer (BD Biosciences) and FlowJo software (Tree Star).

Quantitative PCR

Total RNA from purified CD4 T cells was extracted, using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was performed using AffinityScript qPCR cDNA Synthesis Kit and using oligo dT primers according to the manufacturer's instructions (Agilent Technologies). Quantitative PCR (qPCR) was performed using iTaq SYBR Green Supermix containing Rhodamine X (Bio-Rad Laboratories). cDNA was amplified using a StepOnePlus machine (Applied Biosystems). Relative mRNA expression was calculated using the $\Delta\Delta C_t$ (cycle threshold) method. Data were normalized to the housekeeping gene, S9. Results were expressed as a fold change over the control.

Protein extraction and ELISA

To measure HB-EGF produced by CD4 T cells, following incubation of the cells with OVA, cells were purified by MACS and cultured at the concentration of 5×10^6 cells/ml in ultralow binding plates with PMA (75 ng), ionomycin (1 µM), and GM6001 (25 µM). After 4 h, cells were harvested, and supernatant from the medium was collected and stored at -80°C for further analysis by ELISA. Cell lysate was prepared using lysis buffer (NaCl [250 mM], EDTA [5 mM], NaF [50 mM], Na₃VO₄ [1 mM], NaN₃ [0.02%], sodium deoxycholate [0.5%], Tris-Cl [50 mM], NP-40 [1%], PMSF [1 mM], and proteinase inhibitor mixture [Sigma-Aldrich]). HB-EGF was quantified using a mouse HB-EGF ELISA kit (USCN Life Science).

Assessment of respiratory system mechanics and airway responsiveness to methacholine

Mice were sedated with xylazine hydrochloride (8 mg/kg, i.p.) and anesthetized with sodium pentobarbital (50 mg/kg, i.p.). A tracheostomy was performed using an 18-gauge cannula, which was subsequently used to connect the mouse to a small animal ventilator (flexiVent FX1; SCIREQ, Montreal, QC, Canada). Mice were ventilated with a tidal volume of 10 ml/kg, maximum inflation pressure of 30 cm H₂O, a positive end expiratory pressure of 3 cm H₂O, and a frequency of 150 breaths/min. Rocuronium pentabromide (0.2 mg/kg, i.p.) was administered to induce muscle paralysis and prevent spontaneous breathing. Airway responsiveness was measured by exposing mice to increasing concentrations of methacholine in PBS (0, 6.25, 12.5, 25, and 50 mg/ml) using an inline nebulizer. The software took six

measurements per dose and used the constant phase model to compute changes in respiratory system mechanics.

BAL fluid and serum preparation

Following the assessment of respiratory system mechanics, 1 ml of sterile PBS was injected into the lungs through the tracheal cannula, and the recovered fluid was kept on ice. BAL fluid was centrifuged at 1500 rpm $\times$ g for 5 min at 4°C, and the supernatant was collected for analysis at -80°C. The cell pellet was resuspended in 500 μ l sterile PBS. Cells were counted using a hemocytometer. Cells were mounted on slides using a cytocentrifuge (Shandon) and stained with Diff-Quik (Medical Diagnostics). Differential cell counts were evaluated by identifying 300 cells per slide based on morphology. Blood was collected via cardiac puncture and transferred to a 1.5 ml Eppendorf tube. After centrifugation at 3000 rpm $\times$ g for 10 min at room temperature with slow deceleration, the serum was removed and stored at -80°C.

Cytokine/chemokine multiplex bead assay of BAL

Cytokine levels in BAL were measured using a MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead-based kit, and data were acquired on a Luminex FLEXMAP 3D system (MilliporeSigma).

Histological analysis

Paraffin-embedded lung sections were stained with Periodic acid-Schiff (PAS). The staining was performed with a PAS staining kit (Merck, Darmstadt, Germany). Quantification of goblet cell hyperplasia was performed by counting the number of cells and standardizing for airway size by dividing the count by the perimeter of the basement membrane. The perimeter of the basement membrane was measured with the Image J software. Lung inflammation was scored for cellular infiltration around the airways: 0, no infiltrates; 1, one layer of inflammatory cells; 2, two layers of inflammatory cells; 3, three layers of inflammatory cells; and 4, four and more layers of inflammatory cells.

Immunoblotting and immunoprecipitation

CD4 T cells were purified by MACS and stimulated with anti-CD3 and anti-CD28 for 3 d. Cells were lysed in RIPA lysis buffer (1% NP-40, 150 mM NaCl, 0.1% NaDodSO₄, 0.5% sodium deoxycholate, 50 mM Tris [pH 8], and 1 mM EDTA), supplemented with antiphosphatase and protease inhibitor mixture (Sigma-Aldrich). Protein A/G beads (Life Technologies) and either anti-HB-EGF Ab or IgG2a isotype control were added to 500 μ g of protein extract. Samples were incubated overnight at 4°C with gentle agitation. Immunoprecipitates were washed three times with lysis buffer and boiled in 2 $\times$ Laemmli buffer. Samples were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with BSA (5%) and incubated overnight at 4°C with either anti-HB-EGF or anti-Bcl-6 primary Abs. Membranes were incubated with HRP-conjugated secondary Abs for 1 h at room temperature. Signal was detected using Clarity ECL kit (Bio-Rad Laboratories) and visualized by ChemiDoc MP System (Bio-Rad Laboratories).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed using an EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (MilliporeSigma), following the manufacturer's instruction. CD4 T cells were purified by MACS from spleens of HB-EGF^{lox/lox}CD4CreER^{T2} mice and stimulated with anti-CD3 (3 μ g/ml) and anti-CD28 (1 μ g/ml) for 24 h. CD4 T cells were treated with either 4-OHT (Sigma-Aldrich) dissolved in methanol (1.5 μ g/ml) or the vehicle for 48 h. 1.5×10^6 live cells were fixed with 1% formaldehyde and lysed with lysis buffer. Cell lysates were sonicated using a 150T Digital Sonic Dismembrator (Thermo Fisher Scientific). Immunoprecipitation was performed with either rabbit anti-mouse Bcl-6 Ab, 5 μ g (Santa Cruz Biotechnology), or normal rabbit IgG (Cell Signaling Technology). Purified DNA was subjected to qPCR for IL-5 binding site for Bcl-6 using the following primer sequence: 5'-TGGGCCTTACTTCTCCGTGTAAC-3' (forward), 5'-CTCCAGTGACCTGATACCTGAAT-3' (reverse) (10). The fold enrichment was quantified by normalizing the specific Ab-enriched chromatin against the nonspecific IgG-enriched chromatin.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad). All data are expressed as means $\pm$ SEM with at least two independent experiments. To test for statistical differences between

more than two groups, one-way ANOVA with Tukey post hoc test and two-way ANOVA with Bonferroni posttest were applied. To compare two groups, Student *t* tests were performed. The *p* values <0.05 were considered significant.

Results

Stimulation of CD4 T cells upregulates HB-EGF expression

The OVA transgenic DO.11.10 CD4 T cells expressed mRNA for EGF, HB-EGF, amphiregulin, epiregulin, β -cellulin, TGF- α , and neuregulins 1–4, in the absence of stimulation. Following stimulation with OVA, the mRNA for HB-EGF increased, whereas those for the other tested EGFR ligands either did not change or were significantly lower than in unstimulated cells (Fig. 1A). To confirm that HB-EGF mRNA expression could be induced in nontransgenic mice, purified CD4 T cells from the spleen of BALB/c mice were stimulated with anti-CD3 and anti-CD28 Abs (Fig. 1B). Additionally, splenocytes from in vivo OVA-sensitized BALB/c mice were challenged with OVA for 4, 6, and 8 d in vitro, and subsequently, CD4 T cells were purified (Fig. 1C). In both instances, HB-EGF mRNA expression was significantly increased compared with control.

We next quantified HB-EGF production at the protein level in both the cell lysates and supernatant of DO.11.10 CD4 T cells after OVA stimulation. Because HB-EGF release occurs following the activation of MMPs (20–23), we also examined if release was affected by GM6001, a broad-spectrum MMP inhibitor. We additionally used PMA and ionomycin stimulation as a control to measure sHB-EGF because the stimuli induces shedding of its N-terminal domain from the cell membrane, possibly by activating MMPs (24–26). The HB-EGF concentration in cell lysates from OVA-stimulated CD4 cells was significantly higher than in control unstimulated cells; PMA and ionomycin did not affect intracellular HB-EGF synthesis (Fig. 1D). The medium from CD4 T cell cultures after 4 h demonstrated that HB-EGF release was induced by PMA and ionomycin while inhibited by GM6001 (Fig. 1E). The biased differentiation of T_H2 was confirmed by increased GATA3 mRNA expression in the OVA-stimulated condition (Supplemental Fig. 1A). We next examined the effect of inhibition on T_H2 differentiation on HB-EGF synthesis. OVA-stimulated CD4 T cells synthesized less HB-EGF when stimulated with anti-IL-4-neutralizing Ab (Fig. 1F). We checked the efficacy of anti-IL-4 in reducing T_H2 differentiation of the cells by examining the concomitant expression of IL-5. Anti-IL-4 treatment of cells reduced IL-5 expression from 12.8 ± 1.49 to 8.9 ± 0.6 ($p = 0.037$, $n = 3$). To safely conclude that increased HB-EGF synthesis is induced by OVA protein and not its LPS contamination, we used OVA peptide instead, which similarly resulted in increased HB-EGF protein synthesis in the cell lysate (Supplemental Fig. 1B).

CD4 T cells deficient in HB-EGF mount an attenuated airway inflammation and hyperresponsiveness

To investigate the immuno-physiological role of HB-EGF synthesized by CD4 T cells, a novel strain of mice, HB-EGF^{lox/lox}CD4CreER^{T2}, was generated. In the CD4CreER^{T2} system, Cre recombinase is fused to a mutant form of estrogen receptor ligand binding domain, which binds to tamoxifen preferentially. Cre recombinase expression is under the control of the promoter of CD4 gene and is sequestered in the cytoplasm in the basal state upon expression, whereas it translocates to the nucleus in the presence of tamoxifen (27). The efficiency of HB-EGF knockout in CD4 T cells purified from spleen was assessed by measuring the mRNA expression and qPCR (Supplemental Fig. 2A). Mice were induced to develop acute allergic asthma in response to OVA, although HB-EGF knockout in CD4 T cells was induced between immunization and

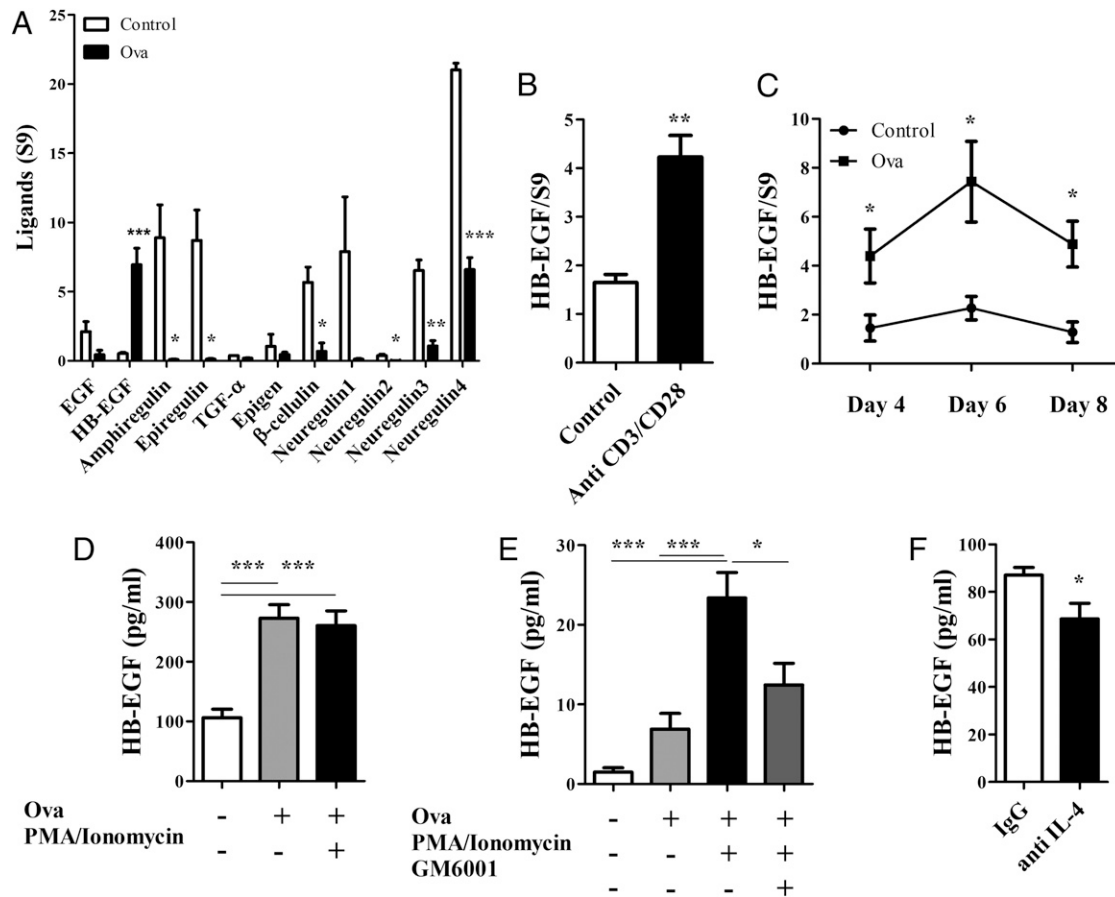


FIGURE 1. Stimulation induces increased expression of HB-EGF by CD4 T cells. **(A)** Splenocytes from DO.11.10 mice were stimulated with OVA in vitro for 3 d, and purified CD4 T cells were examined for expression of EGFR ligands by qPCR ($n = 3-4$). **(B)** Purified CD4 T cells from BALB/c mice were stimulated with anti-CD3 and anti-CD28 Abs for 3 d. HB-EGF mRNA expression was examined by qPCR ($n = 3$). **(C)** Splenocytes from OVA-sensitized BALB/c mice were stimulated with OVA in vitro for 3 d. CD4 T cells were purified and mRNA expression was measured by qPCR ($n = 4-5$). DO.11.10 splenocytes were stimulated with OVA in vitro for 3 d. CD4 T cells were purified and treated with PMA, ionomycin, and GM6001 for 4 h. Data are means $\pm$ 1 SE. Student t test; $*p < 0.05$, $**p < 0.01$. HB-EGF protein was measured **(D)** in cell lysate ($n = 5-6$) and **(E)** supernatant ($n = 5-6$). Data are mean $\pm$ 1 SE. One-way ANOVA with Tukey posttest; $*p < 0.05$, $***p < 0.001$. **(F)** Splenocytes from DO.11.10 mice were stimulated with OVA for 7 d with either anti-IL-4 Ab or isotype control. Purified CD4 T cells were tested for HB-EGF protein expression by ELISA ($n = 4$). Data are means $\pm$ 1 SE. Student t test; $*p < 0.05$.

challenge phases (Supplemental Fig. 2B). Goblet cell hyperplasia and lung inflammation was lower in HB-EGF knockout mice (Fig. 2A–C). Reduced eosinophils in the BAL and lung tissue from HB-EGF knockout animals were confirmed by differential cell count and flow cytometry (CD11c⁺, CD11b⁺, Siglec-F⁺), respectively (Fig. 2D, 2E).

Reduced immune response to allergens and inflammation may attenuate AHR in the acute model of asthma (28, 29). Hence, we examined respiratory mechanical properties in response to increasing doses of methacholine, which demonstrated that knocking out HB-EGF in CD4 T cells attenuates AHR in the peripheral lung, shown as tissue elastance (H) (Fig. 2F). To examine the possible effect of tamoxifen on airway inflammation and hyper-responsiveness, wild type C57BL/6J mice were treated with the same protocol. Eosinophilic inflammation and AHR were quantified as unchanged under tamoxifen administration (Supplemental Fig. 2C, 2E).

Although allergic asthma is classically identified as a T_H2-associated disorder, a subgroup of severe asthmatics develop a mixed T_H2 and T_H17 response (30, 31). Hence, we examined the regulatory effect of HB-EGF expression by CD4 T cells on IL-17A expression in vivo. Upon induction of the acute allergic airway response, the number of IL-17A-expressing CD4 T cells was not affected by HB-EGF deficiency measured by flow

cytometry. Moreover, the level of IL-17A cytokines in the BAL from knockout mice was not different (Supplemental Fig. 3A, 3B).

HB-EGF synthesized by CD4 T cells in the lung modulates IL-5 expression

Eosinophilia has been associated with effector functions of T_H2 immune response. IL-5 is essential for eosinophil differentiation and trafficking (19). Humanized mAb against IL-5, mepolizumab, and reslizumab, reduce sputum eosinophils and improve airway function in patients with eosinophilic asthma (17, 32, 33). To examine the role of HB-EGF by CD4 T cells in IL-5 synthesis, HB-EGF^{lox/lox}CD4CreER^{T2} mice were sensitized and challenged with OVA, and IL-5 protein in the lung was measured by flow cytometry and by a multiplex assay. The frequency and total cell number of IL-5-expressing CD4 T cells were reduced in the lung of mice with HB-EGF-deficient CD4 T cells (Fig. 3B, 3C). The IL-5 cytokine in BAL was present in lower levels as well (Fig. 3D). BAL IL-5 was not affected under tamoxifen administration in wild type C57BL/6J mice (Supplemental Fig. 2D).

IL-5 mRNA expression by T_H2 cells is modulated via HB-EGF interaction with Bcl-6

The HB-EGF C-terminal domain of pro-HB-EGF is shown to bind to and repress Bcl-6 function in nonimmune cells (5, 7). Bcl-6 is a

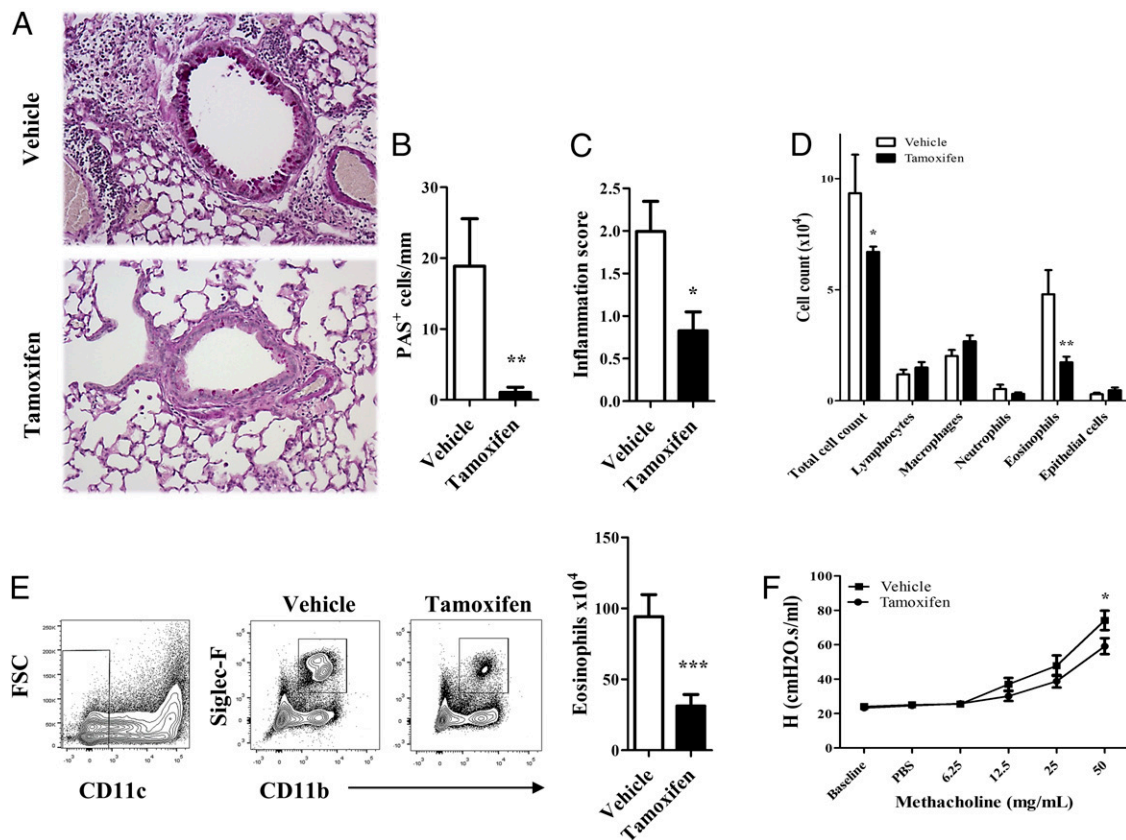


FIGURE 2. HB-EGF synthesized by CD4 T cells positively contributes to eosinophilic inflammation and AHR in an acute model of asthma. (A) Representative PAS-stained lung sections from OVA-sensitized and challenged HB-EGF^{lox/lox}CD4CreER^{T2} mice. (B) Goblet cell hyperplasia and (C) inflammation ($n = 5$) were quantified. (D) Differential cell count was performed for inflammatory cells in BAL ($n = 7$). (E) Total cell number of eosinophils in the lung tissue was assessed by flow cytometry ($n = 6$). Gating strategy represents CD11b⁺ and Siglec-F⁺ population gated on viable CD11c⁻ cells. (F) OVA-sensitized and challenged HB-EGF^{lox/lox}CD4CreER^{T2} mice were examined for lung tissue elastance (H) in response to methacholine 48 h after the last challenge ($n = 6-7$). Data are shown as the mean $\pm$ SE. Student t test and two-way ANOVA with Bonferroni multiple comparison; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

transcriptional repressor of the IL-5 gene in T_H2 cells (9). We aimed to investigate whether HB-EGF in CD4 T cells could induce transcription of the IL-5 gene by antagonizing the effects of Bcl-6. In vitro HB-EGF knockdown in anti-CD3- and anti-CD28-stimulated CD4 T cells resulted in reduced IL-5 mRNA expression (Fig. 4A, 4B). Coimmunoprecipitation of HB-EGF and Bcl-6 confirmed their interaction in CD4 T cells stimulated with anti-CD3 and anti-CD28 Abs (Fig. 4C). Deficiency of HB-EGF in CD4

T cells enhanced Bcl-6 binding to the IL-5 gene assessed by ChIP assay (Fig. 4D).

Bcl-6 expression by CD4 T cells regulates IL-5 synthesis and allergic airway response

To determine whether Bcl-6 is involved in allergic airway inflammation and dysfunction via repressing IL-5 synthesis, OVA-sensitized and challenged Bcl-6^{lox/lox}CD4Cre mice were shown

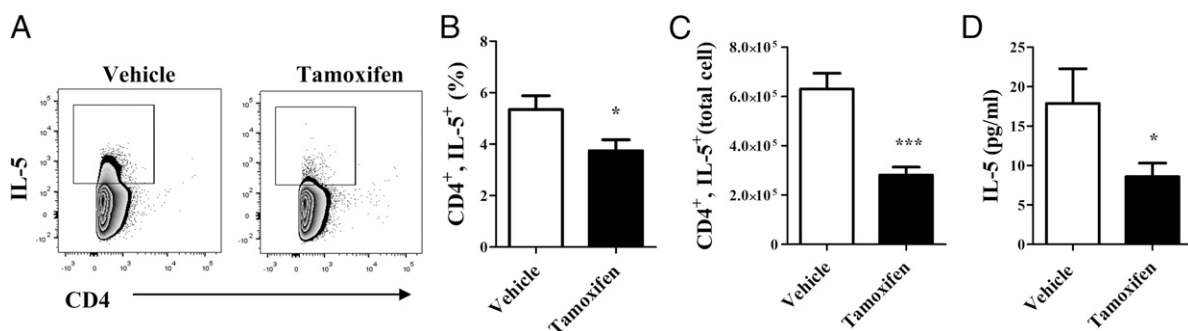


FIGURE 3. HB-EGF is essential for IL-5 expression by CD4 T cells. (A) Flow cytometric analysis of IL-5-expressing CD4 T cells presented as (B) frequency (%) and (C) total cell number in the lung of OVA-sensitized and challenged HB-EGF^{lox/lox}CD4CreER^{T2} mice ($n = 6$). eFluor780⁻ viable cells were gated for CD11c⁻ and CD4⁺ population. IL-5⁺ cells were gated within the CD4 T cell population. (D) IL-5 protein was quantified in BAL from OVA-sensitized and challenged HB-EGF^{lox/lox}CD4CreER^{T2} mice ($n = 7$). Data are mean $\pm$ 1 SE. Student t test; * $p < 0.05$, *** $p < 0.001$.

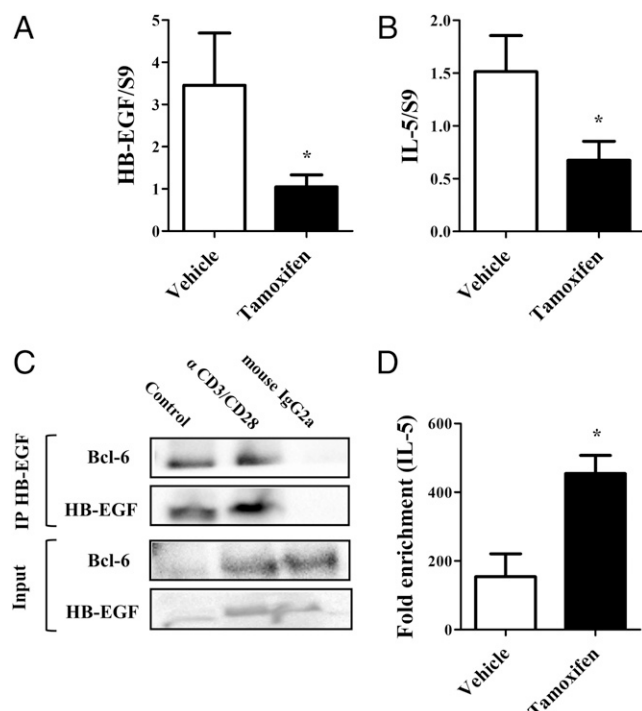


FIGURE 4. Intracellular HB-EGF regulates IL-5 expression by CD4 T cells via interacting with Bcl-6. **(A)** Knocking out CD4 T cell HB-EGF in vitro by 4-OHT. CD4 T cells from HB-EGF^{lox/lox}CD4CreER^{T2} mice spleens were stimulated with anti-CD3 and anti-CD28 Abs in the presence of either 4-OHT or vehicle ($n = 8$). **(B)** Knocking out CD4 T cell HB-EGF in vitro reduced IL-5 mRNA expression ($n = 8$). **(C)** CD4 T cells isolated from spleen of wild type mice were stimulated with anti-CD3 and anti-CD28 for 3 d. Immunoprecipitation was performed on the cell lysate using Ab against the C-terminal domain of HB-EGF. Samples were analyzed by immunoblotting for HB-EGF and Bcl-6 ($n = 3$). **(D)** Assessing Bcl-6 binding to the IL-5 gene by ChIP assay followed by qPCR. To prepare samples, CD4 T cells, purified from spleen of HB-EGF^{lox/lox}CD4CreER^{T2} mice, were stimulated with anti-CD3 and anti-CD28 in the presence of either 4-OHT or vehicle. Data are presented as fold enrichment of the IL-5 gene immunoprecipitated by anti-Bcl-6 Ab against IgG-enriched chromatin ($n = 3-4$). Data are shown as the mean $\pm$ 1 SE. Student t test and two-way ANOVA with Bonferroni multiple comparison; $*p < 0.05$.

to develop greater eosinophilia (Fig. 5A) and IL-5 levels in the serum (Fig. 5B) as well as AHR (Fig. 5C).

HB-EGF expression by T_H2 cells from asthmatic patients is greater than healthy subjects

We examined in vitro HB-EGF expression by human T_H2 cells from healthy donors. CD4 T cells stimulated with anti-CD3 and

anti-CD28 had a higher HB-EGF expression in IL-5-expressing populations compared with non-IL-5-expressing cells (Fig. 6A). To determine the contribution of CD4 T cell synthesized HB-EGF in allergic asthma, we examined CD4 T cells from asthmatics showing a higher frequency of T_H2 cells expressing IL-5 and HB-EGF compared with healthy subjects after in vitro stimulation (Fig. 6B). The proposed mechanism by which HB-EGF modulates T_H2 immunological function is shown in illustrative Fig. 7.

Discussion

This study elucidates a novel mechanism regulating T_H2 immunological function in the airway allergic response. CD4 T cells increase HB-EGF synthesis in response to various activation stimuli. HB-EGF synthesized by CD4 T cells enhances IL-5 gene expression, contributing to eosinophilia and possibly AHR in a model of acute allergic asthma. Our data suggest that the HB-EGF immunoregulatory mechanism occurs via the interaction with and suppression of Bcl-6 transcriptional repressive function for IL-5 gene expression.

Expression of EGFs by CD4 T cells has long been documented. Amphiregulin synthesis by CD4 T cells has been attributed to the function of tissue repair when produced by T_H2 cells and the induction of pulmonary fibrosis when produced by memory T_H2 cells (3, 34). Although HB-EGF synthesis by human CD4 T cells has been reported (2), there is a lack of studies investigating its immuno-physiological function. We demonstrated that DO.11.10 CD4 T cells, bearing the OVA-specific TCR, increased both mRNA and protein expression of HB-EGF in vitro after stimulation with OVA, whereas mRNA expression for other EGFR ligands that we examined did not follow the same pattern. Using GM6001 as a broad-spectrum MMP inhibitor, we confirmed the involvement of MMPs in the cleavage and release of HB-EGF from CD4 T cells. PMA and ionomycin, shown to induce HB-EGF shedding from the cell membrane (24–26), was used as a control for enhanced HB-EGF release without affecting the synthesis. mRNA expression for the signature transcription factors of T_H1, T_H2, T_H17, and T_{reg} suggested increased expression of HB-EGF mRNA expression correlated with a dominant expression of GATA3 as an indicator of T_H2 differentiation. Neutralization of IL-4 signaling during CD4 T cell stimulation by OVA in vitro reduced HB-EGF synthesis, which suggests a mechanistic role for increased HB-EGF expression by T_H2 cells. The partial, albeit significant reduction in T_H2 differentiation, measured as IL-5 expression, after anti-IL-4 Ab administration suggests that IL-4 neutralization was not potent enough. Hence, a more effective method of inhibition of IL-4 signaling may further reduce HB-EGF expression. Alternatively, HB-EGF expression by CD4 T cells may be influenced by other cytokines, for which we currently have no evidence.

FIGURE 5. Bcl-6 regulates the response to allergens in acute allergic asthma. In an acute model of asthma induced by OVA, **(A)** eosinophilic inflammation in BAL and **(B)** the IL-5 level in the serum was measured in Bcl6^{lox/lox}CD4Cre mice deficient for Bcl-6 in CD4 T cells ($n = 3-5$). **(C)** AHR was measured as tissue elastance (H) ($n = 3-4$). Data are shown as the mean $\pm$ 1 SE. Student t test and two-way ANOVA with Bonferroni multiple comparison; $*p < 0.05$, $**p < 0.01$. UD, undetectable.

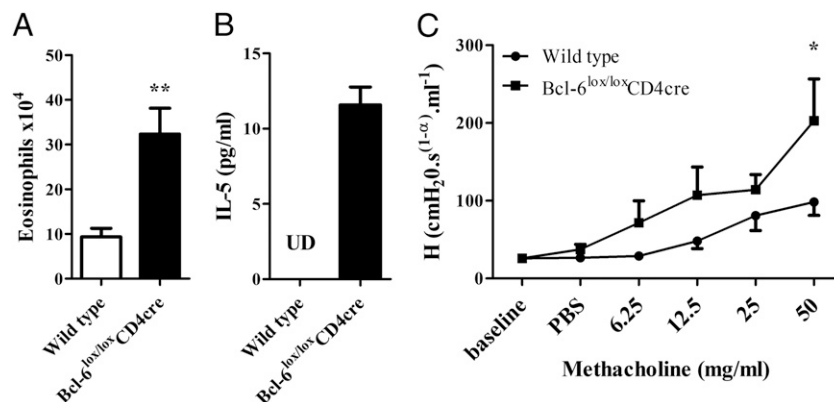
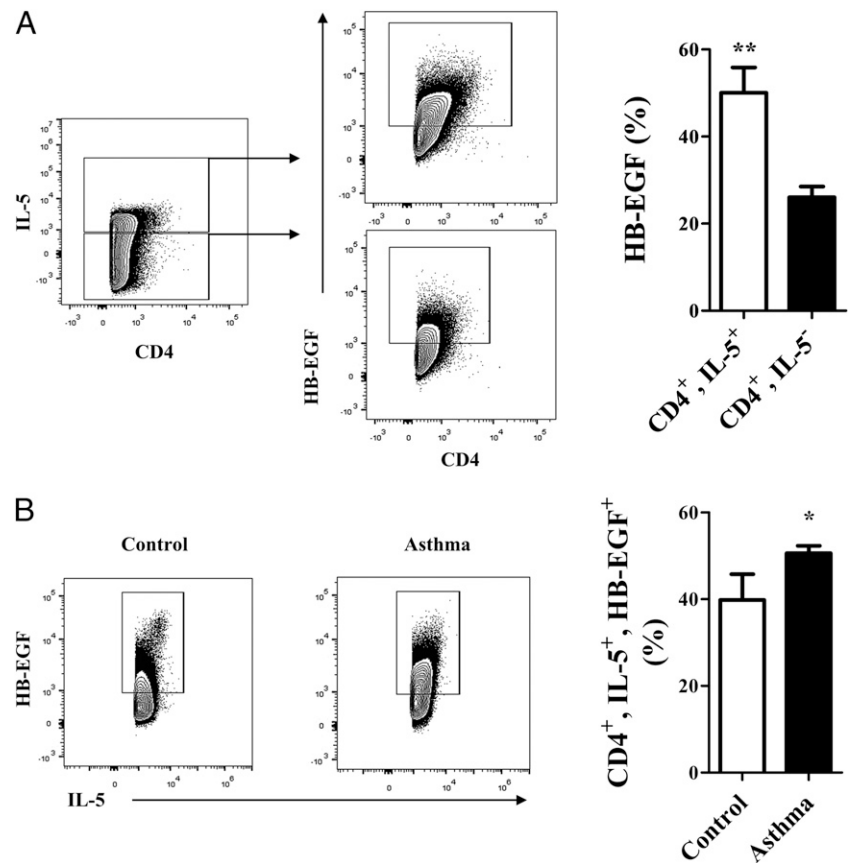


FIGURE 6. T_H2 cells from asthmatic patients express more HB-EGF. **(A)** CD4 T cells were purified from PBMC from healthy donors and stimulated with anti-CD3 and anti-CD28 for 3 d. HB-EGF expression was assessed in both IL-5–positive and –negative CD4 T cell populations by flow cytometry ($n = 5$). **(B)** CD4 T cells from PBMC from healthy and asthmatic subjects were stimulated with anti-CD3 and anti-CD28 for 3 d. HB-EGF expression by IL-5–expressing CD4⁺ T cell populations was measured by flow cytometry ($n = 5–6$). Data are shown as the mean $\pm$ 1 SE. Student *t* test and two-way ANOVA with Bonferroni multiple comparison; * $p < 0.05$, ** $p < 0.01$.



HB-EGF mRNA expression by stimulated CD4 T cells was further confirmed in nontransgenic wild type mice.

Allergic asthma has been long associated with eosinophilic inflammation driven and sustained by T_H2 cells. The pathophysiological role of HB-EGF by CD4 T cells in allergen-induced airway inflammation and AHR was examined using the conditional inducible HB-EGF knockout mice, HB-EGF^{lox/lox}CD4CreER^{T2}. Knocking out HB-EGF in CD4 T cells by tamoxifen following sensitization resulted in modest effects on responses to inhaled

methacholine. We observed reduced eosinophilic inflammation and goblet cell hyperplasia in the lung, which was associated with a reduced level of IL-5 synthesis by CD4 T cells deficient in HB-EGF, observed both in vitro and in vivo. However, knocking out HB-EGF in CD4 T cells did not affect IL-17A synthesis, which is a hallmark of allergic asthma with a mixed phenotype of T_H2 and T_H17 . AHR in the peripheral airways, reflected in dynamic tissue elastance (H), was also modestly reduced. Tamoxifen did not affect allergen-induced eosinophilic inflammation, IL-5, or AHR assessed

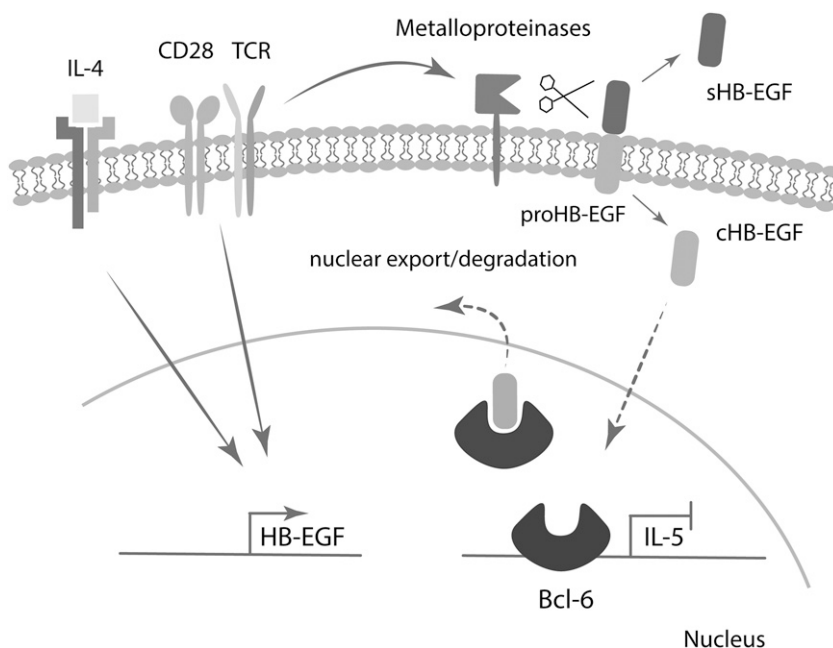


FIGURE 7. Schematic illustration of expression and immunoregulatory function of CD4 T cell HB-EGF. Stimulation of CD4 T cells results in increased expression of HB-EGF. There is no evidence for the differential role of TCR stimulation and costimulation in HB-EGF expression. Neutralization of IL-4 results in decreased HB-EGF synthesis by CD4 T cells, which suggests a partial role for IL-4 on HB-EGF expression. Activation of CD4 T cells induces the activation of metalloproteinases (41), which in turn may cleave HB-EGF from the cell membrane to release sHB-EGF into the extracellular space and cHB-EGF in the cytoplasm. cHB-EGF translocates into the nucleus and binds to and inhibits the Bcl-6 repressive effect on IL-5 gene expression by facilitating Bcl-6 export from nucleolus.

in wild type mice treated similarly to HB-EGF^{lox/lox}CD4CreER^{T2} animals.

Eosinophils have an important role in allergic asthma. Eosinophils may adversely affect airway mechanics by producing lipid mediators, such as cysteinyl leukotrienes, major basic protein, eosinophil peroxidase, and IL-13. Furthermore, in long term and chronic sensitization, eosinophils may contribute to the severity of decreased lung function by inducing airway remodeling, hence increasing the dynamic stiffness of pulmonary tissues (18). However, the relationship between eosinophilic airway inflammation and AHR is not straightforward. IL-5-deficient mice do not develop eosinophilia and AHR in the pulmonary allergic reaction to OVA (35). Two different transgenic mice deficient in eosinophils have resulted in contradictory conclusions showing either no role or inducing a role in allergic AHR (36, 37). Eosinophils are important for effective T_H2 responses so that prolonged deficiency impairs responses and inhibits AHR in response to an allergen challenge (38). The acute neutralization of IL-5 in mice and also a single i.v. administration of anti-IL-5 mAb prior to an allergen challenge in humans, although reducing allergen-induced eosinophilia, does not prevent AHR (39, 40). The duration or magnitude of the inhibition of eosinophilia in the current experiment may have been insufficient to have a major impact on AHR. Eosinophil depletion by humanized monoclonal Abs against IL-5 have been successful in reducing exacerbation rates and allowing a reduction in corticosteroid use in eosinophilic asthma (17, 32, 33).

The Bcl-6 repressive function can be reversed by its binding to the cytoplasmic domain of pro-HB-EGF (5, 7). Bcl-6 has a binding site on the IL-5 gene, and CD4 T cells deficient in Bcl-6 synthesize higher levels of IL-5 (9, 12). In this study, we attempted to confirm the interaction between HB-EGF and Bcl-6 in CD4 T cells as it is shown in nonimmune cells. Coimmunoprecipitation of HB-EGF with Bcl-6 in wild type CD4 T cells together with increased Bcl-6 binding to the IL-5 gene when CD4 T cells are deficient in HB-EGF suggests a mechanism by which HB-EGF regulates IL-5 synthesis and, therefore, eosinophilia. In agreement with the inhibitory interaction of HB-EGF with Bcl-6, Bcl-6^{lox/lox}CD4Cre mice sensitized and challenged with OVA developed higher eosinophilia, IL-5 production, and AHR.

To determine if increased HB-EGF expression had a similar biological significance in human to the mouse, we showed that IL-5-producing CD4 T cells express more HB-EGF compared with the IL-5-negative population. A comparison of asthmatics with healthy subjects revealed a higher frequency of the T_H2 cells in the peripheral blood expressing IL-5 that also synthesized HB-EGF. It would be of interest to investigate whether this increased expression of HB-EGF is due to aberrant genetic or epigenetic properties of CD4 T cells in asthma. The asthmatic subjects were on medications that included inhaled and/or oral corticosteroids, and we cannot exclude a possible effect on the relationship between HB-EGF and IL-5 in their CD4 cells. However, cells were studied 3 d after harvesting, at which time drug effects would have been absent.

Our data indicate that HB-EGF synthesized by T_H2 cells has an important contributory role in allergen-induced pulmonary eosinophilia and AHR, potentially by augmenting IL-5 synthesis. The evidence suggests a mechanism of increased IL-5 production is through the interaction of HB-EGF with Bcl-6, thereby inhibiting its repressive action on IL-5 gene expression. The novel immune regulatory role of HB-EGF synthesized by CD4 T cells provides a more complete understanding of the regulation of T_H2 immunological function, which may provide new insight into the therapeutic modulation of allergic immune responses.

Disclosures

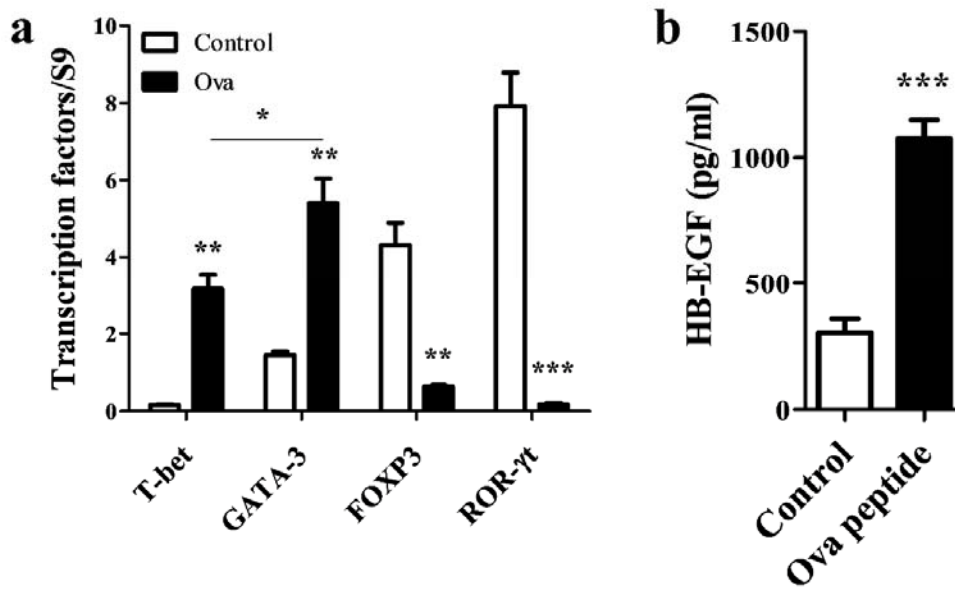
The authors have no financial conflicts of interest.

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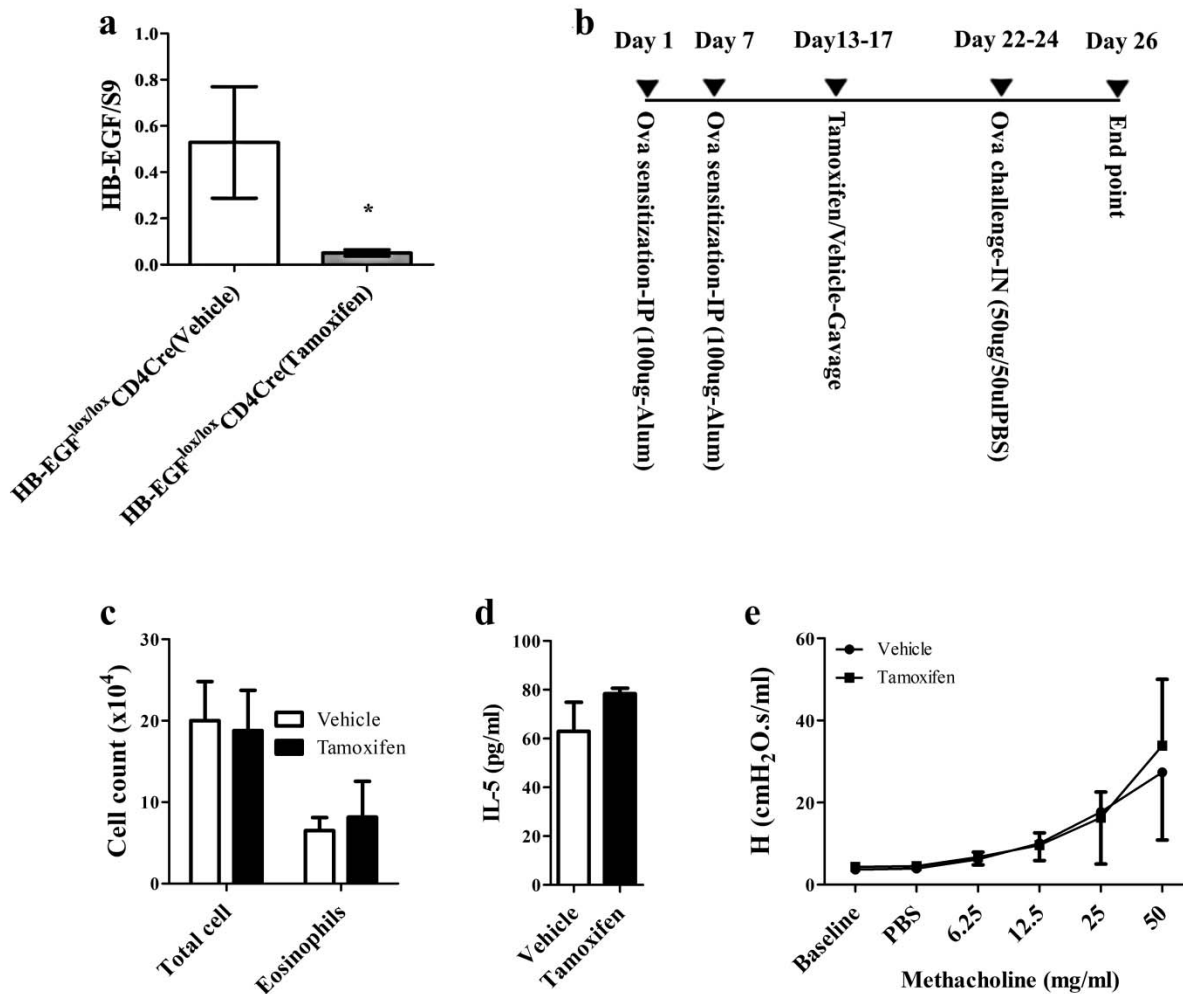
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Supplementary figure. 1



Supplementary figure 1. (a) The differentiation pattern of CD4 T cells from DO.11.10 mice stimulated with Ova *in vitro* based on mRNA expression for signature transcription factor of T_H1 (T-bet), T_H2 (GATA-3), T_H17 (ROR- γ t) and T regulatory cells (FOXP3) measured by qPCR(n=3). (b) HB-EGF synthesis by CD4 T cells from DO.11.10 mice stimulated with Ova peptide, 10 μ g/ml, *in vitro*. Splenocytes were stimulated with Ova peptide for 3 days. CD4 T cells were purified and HB-EGF protein in the cell lysate was measured by ELISA (n= 3-4). Data are shown as the mean $\pm$ 1SE. One-way ANOVA with Tukey posttest; *P<0.05, **P<0.01, ***P<0.001.

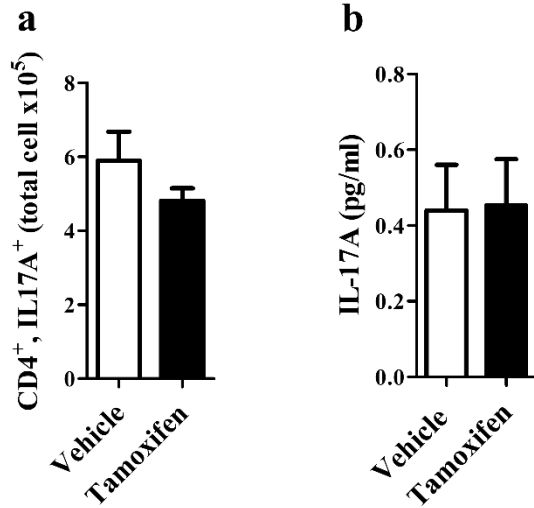
Supplementary figure. 2



Supplementary figure 2. HB-EGF knockdown induced by tamoxifen. (a) HB-EGF^{lox/lox}CD4Cre mice were treated with either tamoxifen or vehicle by gavage for 5 consecutive days. After 5 days the efficiency of HB-EGF knockdown in purified CD4 T cells from spleen was assessed by RT-qPCR (n=7). (b) The schematic representation of Ova sensitization and challenge. To study the effect of tamoxifen on acute allergic response in wild type mice, C57BL/6J mice were sensitized with Ova, treated with tamoxifen and subsequently challenged with Ova. (c) total cell count and eosinophilic inflammation were assessed in BAL (n=5). (d) IL-5 was measured in BAL by ELISA (n=3). (e) AHR was measured as tissue

elastance in response to increasing doses of methacholine (n=5-6). Data are mean $\pm$ 1SE. Student's t-test; *P<0.05.

Supplementary figure. 3



Supplementary figure 3. The regulatory effect of HB-EGF expressed by CD4 T cells on IL-17A synthesis. Using flow cytometry, in the lung of Ova sensitized and challenged HB-EGF^{lox/lox}CD4Cre mice (a) number of IL-17A expressing CD4 T cells (n=6) was quantified. eFluor780⁻ viable cells were gated for CD11c⁻, CD4⁺ population. IL-17A⁺ cells were gated within the CD4 T cell population. (b) IL-5 protein was measured in BAL from Ova sensitized and challenged HB-EGF^{lox/lox}CD4Cre mice (n=7).

Supplementary table 1. Demographic information of asthmatic patients.

Patients	Sex	Age	FEV ₁ (%predicted)	Medications
1	Male	54	46%	Salbutamol; PRN* Mometasone (400µg)/Formoterol (10µg); BID*
2	Male	63	29%	Montelukast (10mg); daily Ciclesonide (400µg); BID Mometasone (400µg)/Formoterol (10µg); BID Salbutamol; PRN Prednisone (30mg); daily Indacaterol (75µg); daily Glycopyrronium (50mg); daily
3	Female	54	66%	Budesonide (400µg)/Formoterol (12µg); BID Salbutamol; PRN Tiotropium bromide(2.5mg)
4	Female	55	63%	Salbutamol; PRN Fluticasone (500µg)/Salmeterol (50µg); BID
5	Female	50	69%	Tiotropium bromide(2.5mg) Salbutamol; PRN Ciclesonide (400µg); BID Fluticasone (500µg)/Salmeterol (50µg); BID

PRN: as needed
BID: twice a day