

The eosinophil surface receptor epidermal growth factor–like module containing mucin-like hormone receptor 1 (EMR1): A novel therapeutic target for eosinophilic disorders

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Background: Although several novel agents are currently in clinical trials for eosinophilic disorders, none has demonstrated efficacy in reducing blood and tissue eosinophilia in all subjects. Additional approaches are clearly needed.

Objective: We sought to explore the potential of the human eosinophil surface receptor epidermal growth factor–like module containing mucin-like hormone receptor 1 (EMR1) as a therapeutic target for eosinophilic disorders.

Methods: EMR1 expression was assessed in blood and bone marrow specimens from eosinophilic and healthy subjects, cell lines, CD34⁺ cells differentiated *in vitro*, and tissue biopsy specimens by using flow cytometry, quantitative PCR, and immunostaining. Eosinophil targeting by a novel, humanized, afucosylated anti-EMR1 IgG₁ was evaluated *in vitro* by using a natural killer cell–mediated killing assay and *in vivo* in cynomolgus monkeys.

Results: Analysis of blood and bone marrow cells from healthy and eosinophilic donors and *in vitro*–differentiated CD34⁺ cells confirmed restriction of human EMR1 surface and mRNA expression to mature eosinophils. Tissue eosinophils also expressed EMR1. Although EMR1 was highly expressed on eosinophils from all subjects, surface expression was negatively correlated with absolute eosinophil counts ($r = -0.46$, $P < .001$), and soluble plasma levels correlated positively with absolute eosinophil counts ($r = 0.69$, $P < .001$), suggesting modulation of

EMR1 *in vivo*. Nevertheless, afucosylated anti-EMR1 mAb dramatically enhanced natural killer cell–mediated killing of eosinophils from healthy and eosinophilic donors and induced a rapid and sustained depletion of eosinophils in monkeys.

Conclusion: EMR1 expression is restricted to mature blood and tissue eosinophils. Targeting of eosinophils with afucosylated anti-EMR1 antibody shows promise as a treatment for eosinophilic disorders. (J Allergy Clin Immunol 2014;■■■■:■■■■–■■■■.)

Key words: Epidermal growth factor–like module containing mucin-like hormone receptor 1, eosinophil, hypereosinophilic syndrome, mAb therapy, afucosylated antibody, eosinophilia

Eosinophils are multifunctional leukocytes implicated in the pathogenesis of a wide variety of disorders, including asthma, helminth infections, hematologic malignancies, and hypereosinophilic syndromes (HESs).¹ Although glucocorticoids are first-line therapy for many of these disorders, long-term toxicity is common, and resistance can occur. Whereas clinical trials of novel therapeutic agents targeting eosinophils, including mAbs to IL-5 (mepolizumab and reslizumab) and IL-5 receptor (IL-5R) α (benralizumab),^{2,3} have demonstrated the safety of eosinophil depletion in the treatment of various eosinophilic disorders, including eosinophilic asthma, eosinophilic esophagitis, HES, and eosinophilic granulomatosis with polyangiitis, none has shown complete elimination of tissue eosinophils or clinical symptoms. Clearly, additional targeted therapies are needed.

Human epidermal growth factor (EGF)–like module containing mucin-like hormone receptor 1 (EMR1) is a surface receptor of unknown function that belongs to the EGF-7-transmembrane family of G protein–coupled receptors.⁴ Other family members include EMR2 and CD97, molecules involved in modulation of neutrophil activation and leukocyte migration to sites of inflammation.^{5–7} The human ortholog of F4/80, which is expressed on murine monocytes, macrophages, myeloid dendritic cells, and eosinophils,⁸ human EMR1 has been reported to be expressed exclusively on eosinophils⁹ and to be highly expressed in nasal polyps.¹⁰ Therefore EMR1 is an ideal potential target for the treatment of eosinophilic disorders.

In the present study we describe the expression profile of EMR1 in the blood, bone marrow, and tissue of healthy donors and subjects with a variety of eosinophilic disorders. Modulation of the receptor was also explored *ex vivo* and *in vitro*. Finally, the efficacy of an afucosylated chimeric antibody to EMR1 to target eosinophils

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Abbreviations used

ADCC: Antibody-dependent cell-mediated cytotoxicity
 AEC: Absolute eosinophil count
 CHO: Chinese hamster ovary
 ECP: Eosinophil cationic protein
 EDN: Eosinophil-derived neurotoxin
 EGF: Epidermal growth factor
 EMR1: EGF-like module containing mucin-like hormone receptor 1
 EPX: Eosinophil peroxidase
 GM: Geometric mean
 HES: Hypereosinophilic syndrome
 IL-5R: IL-5 receptor
 MBP: Major basic protein
 NIH: National Institutes of Health
 NK: Natural killer
 PBST: PBS with 0.1% Tween 20
 sEMR1: Soluble EMR1

for natural killer (NK) cell-mediated antibody-dependent cell lysis was assessed *in vitro* and *in vivo* in a primate model.

METHODS**Anti-EMR1 mAbs**

Recombinant extracellular domains from human and cynomolgus monkey EMR1 were expressed as Fc fusion proteins in Chinese hamster ovary (CHO) cells. After purification on protein A columns, Fc tags were proteolytically removed by using Factor Xa, and human EMR1 extracellular domain was used for immunization in mice. The mouse hybridoma line 1E7, which expresses high-affinity anti-EMR1 mAb, was grown in Hybridoma SFM media (Invitrogen, Carlsbad, Calif). Afucosylated and fucosylated chimeric 1E7 antibodies with human IgG₁ κ constant regions were expressed in Potelligent CHOK1SV (Biowa/Lonza, Basel, Switzerland).¹¹ Murine and chimeric 1E7 antibodies were purified by means of protein A affinity chromatography.

Study subjects

Eosinophilic subjects (n = 38) underwent detailed clinical and laboratory evaluation as part of an institutional review board–approved clinical protocol to study eosinophilia (NCT00001406) and included subjects with idiopathic HES (n = 18), lymphocytic variant HES (n = 7), helminth infection (n = 4), hypereosinophilia of unknown significance (n = 3), platelet-derived growth factor receptor A (*PDGFRA*)–associated myeloproliferative neoplasm (n = 3), and familial eosinophilia (n = 2). Six subjects were receiving therapy at the time of study but had persistent eosinophilia (geometric mean [GM] absolute eosinophil count [AEC], 2,167/μL; range, 910–13,000/μL). Healthy volunteers were recruited under an institutional review board–approved clinical protocol designed to obtain control subjects for *in vitro* research (NCT00090662). All participants provided written informed consent.

Cell purification

Granulocytes and PBMCs were separated by means of sedimentation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Erythrocytes were lysed by means of hypotonic shock with ice-cold ddH₂O (for granulocytes) or ACK lysing buffer (Quality Biological, Inc, Gaithersburg, Md) (for PBMCs). Individual cell populations were purified by using magnetic bead selection on an Auto-Macs (Miltenyi Biotech, Cambridge, Mass), according to the manufacturer's instructions. Eosinophils and neutrophils were purified from the granulocyte layer by using the Eosinophil Isolation Kit (Miltenyi Biotech). NK cells, CD14⁺ monocytes, and CD34⁺ stem cells were purified from the PBMC layer by using the NK Cell Isolation Kit, anti-CD14 beads, and anti-CD34 beads, respectively (Miltenyi Biotech). Granulocyte purity was determined by counting a minimum of 300 cells on cytospin preparations stained with Diff-Quik

(Siemens Healthcare Diagnostics, Malvern, Pa). The purity of other cells was determined by means of flow cytometry. Purity was greater than 98% for all cell populations studied. Cells for RNA expression analysis were counted and put directly in TriZol Reagent (Invitrogen) at a concentration of 10 × 10⁶/mL.

Human cell lines and culture conditions

Purified peripheral blood eosinophils were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (BioWhittaker, Walkersville, Md), 25 mmol/L HEPES, 2 mmol/L L-glutamine, 10 mmol/L sodium pyruvate, and 50 μg/mL gentamicin (culture medium). The leukemic cell line EOL1 (DSMZ Institute, Braunschweig, Germany), the erythroleukemia cell line K562 (ATCC CCL-243; ATCC, Manassas, Va), and the histiocytic lymphoma cell line U937 (ATCC CRL-1593.2) were maintained in RPMI 1640 medium with 10% FCS at 37°C. AML14.3D10 (ATCC CRL-12079) was maintained in culture medium containing 50 μmol/L β-mercaptoethanol. CHO cells transfected with EMR1 (CHOK1SV) were cultured in CD-CHO (Invitrogen) supplemented with 25 μmol/L L-methionine sulfoximine.

Detection of surface EMR1 by means of flow cytometry

EMR1 expression in bone marrow aspirates and peripheral blood was assessed by using multiparameter flow cytometry with directly conjugated antibodies, as previously described (see the [Methods](#) section in this article's Online Repository at www.jacionline.org for detailed methodology).¹²

Real-time quantitative PCR

Total RNA was extracted from purified cell populations and cell lines with TriZol Reagent (Invitrogen), and cDNA was synthesized from 1 μg of total RNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, Calif), according to the manufacturer's protocol. cDNA from human CD34⁺ cells cultured under conditions to induce mast cell differentiation¹³ and from the mast cell lines HMC-1.1 (lacking KIT D816V), HMC-1.2 (expressing KIT D816V), and LAD2 were provided by Dr Todd Wilson (National Institute of Allergy and Infectious Diseases/National Institutes of Health [NIH]). Approximately 50 ng of RNA equivalent cDNA template was used per well, and real-time amplification was performed in a 96-well plate by using a GeneAmp 7900HT Sequence Detection System (Applied Biosystems). Primers used are provided in the [Methods](#) section in this article's Online Repository. Each sample was run in triplicate, and cycle threshold values were normalized by using 18S cycle threshold values from corresponding samples.

CD34⁺ cell differentiation *in vitro*

Human CD34⁺ cells isolated by means of positive selection from granulocyte colony-stimulating factor–mobilized peripheral blood of 2 healthy donors (provided by Dr Harry Malech, National Institute of Allergy and Infectious Diseases/NIH) were cultured in IMDM (Invitrogen) supplemented with 10% FBS and GM-CSF (1 ng/mL), stem cell factor, Fms-like tyrosine kinase-3 ligand and thrombopoietin (each at 10 ng/mL), IL-5 (25 ng/mL), and IL-3 (20 ng/mL) for 3 days, followed by IL-5 (25 ng/mL) only for another 21 days. Cells were collected on days 4, 6, 8, 10, 12, 15, 18, 21, and 24 of culture, and total RNA and cell lysates were prepared at each time point. Eosinophil differentiation was assessed by using Diff-Quik (Siemens Healthcare Diagnostics) and Fast Green/Neutral Red (Sigma-Aldrich, St Louis, Mo) staining. At 21 days of culture, greater than 60% of the cells were eosinophil like based on morphologic criteria, and greater than 20% were mature eosinophils based on Fast Green staining of cytoplasmic granules. Growth and viability were determined by means of light microscopic evaluation with trypan blue exclusion. Viability remained greater than 95% throughout the 24-day experimental period.

Tissue immunostaining

A 5-mm skin punch biopsy specimen obtained from a patient with HES was bisected. One half of the specimen was fixed in neutral buffered formalin,

routinely processed, sectioned, and stained with hematoxylin and eosin per standard procedures of the Laboratory of Pathology, National Cancer Institute. Immunohistochemical staining for eosinophil peroxidase (EPX) was performed with a mouse anti-mouse mAb (1:500, provided by Dr J. J. Lee). The second portion was snap-frozen for immunostaining with anti-EMR1 antibody (c1E7) by using standard techniques (see the [Methods](#) section in this article's Online Repository for detailed methodology).

Acetone-fixed nasal polyp frozen tissue section slides obtained from Capital Biosciences (female patient, chronic rhinosinusitis with nasal polyps and bronchial asthma, 39 years old) were stained with mouse 1E7-Alexa595 (0.6 $\mu\text{g/mL}$), mouse anti-EPX antibody (diluted 1:200 with 2% BSA with PBS; Abcam, Cambridge, United Kingdom), and/or mouse anti-CD68 (clone KP-1, diluted 1:2; Ventana, Tucson, Ariz) by using standard techniques for visualization by means of immunofluorescence microscopy (see the [Methods](#) section in this article's Online Repository for detailed methodology).

Measurement of soluble receptors in plasma

Plasma levels of soluble EMR1 (sEMR1) were measured by using a sandwich ELISA (MyBioSource, San Diego, Calif). All assays were performed in singlicate, and values were calculated on the basis of a standard curve. The minimal detectable concentration of sEMR1 in plasma was 10 pg/mL.

In vitro killing assay

Purified eosinophils (8×10^4 target) were incubated with autologous NK cells (effector) at an effector/target ratio of 5:1 in 96-well U-bottom plates at 37°C in the presence or absence of 10 $\mu\text{g/mL}$ afucosylated c1E7 or IgG₁ isotype control antibodies. After 4 hours, the assay was stopped by putting the plates on ice and replacing the culture medium with binding buffer (100 μL per well; BD Biosciences, San Jose, Calif). Annexin V-fluorescein isothiocyanate (BD Biosciences) was added, and after 15 minutes of incubation at room temperature, samples were acquired and analyzed on a flow cytometer (LSRII, BD Biosciences). Eosinophils were identified based on their granularity (high side scatter). Antibody-dependent cell-mediated cytotoxicity (ADCC) was determined by gating on Annexin V⁺ target cells.

Administration of anti-EMR1 antibody in nonhuman primates

Male cynomolgus macaques received a single intravenous infusion of c1E7 at either 1 mg/kg ($n = 2$) or 5 mg/kg ($n = 2$) at Bio-Quant (San Diego, Calif) under a protocol approved by Bio-Quant's Institutional Animal Care and Utilization Committee and the attending veterinarian. Whole blood was collected for pharmacokinetic analysis and eosinophil counts at baseline; 5 minutes; 2, 4, and 8 hours; and 1, 2, 4, 8, 15, 22, and 28 days after dosing. The plasma concentration of c1E7 was measured by using a quantitative antigen-based ELISA (see the [Methods](#) section in this article's Online Repository for detailed methods). Complete blood counts and serum chemistries were performed before dosing and on days 8, 28, 42, and 56.

Statistical analysis

Statistical analyses were performed with the nonparametric Mann-Whitney *U* and Wilcoxon tests for unpaired and paired comparisons of group means, respectively, and the Spearman rank test was used for correlation. A *P* value of less than .05 was considered significant for all analyses. Pharmacokinetic analysis was performed by using a noncompartmental model (see the [Methods](#) section in this article's Online Repository).

RESULTS

Surface expression of EMR1 in human subjects is restricted to eosinophils

Flow cytometric analysis was performed with whole blood and bone marrow aspirates from healthy donors and eosinophilic

subjects with mAb 1E7 generated against the recombinant EMR1 extracellular domain to confirm that EMR1 expression in human blood and bone marrow is restricted to eosinophils. As reported previously, by using a different anti-EMR1 mAb,⁹ EMR1 expression was detected exclusively on eosinophils (CD16⁺CD9⁺ granulocytes in blood and IL-5R α ⁺CD16⁺Siglec-8⁺ granulocytes in bone marrow aspirates) in all subjects tested ([Fig 1, A-D](#)). EMR1⁺ cells were also positive for IL-5R α , Siglec-8, CD9, CD15, CD45, and CD49d and negative for CD3, CD14, CD16, CD19, CD34, CD64, and CD203 (data not shown). EMR1 was not detected on mast cells, basophils, monocytes, neutrophils, lymphocytes, the monocytic cell line U937, the erythroleukemic cell line K562, or either of 2 eosinophilic leukemia cell lines, Eol-1 and AMLD14.3D10 ([Fig 1, E](#)), whereas the positive control CHOK1SV cell line (transfected with full-length EMR1 gene) showed uniformly high expression of EMR1 ([Fig 1, E](#)).

Quantitative reverse transcriptase PCR confirmed the presence of *EMR1* mRNA in eosinophils from eosinophilic subjects and healthy control subjects ([Table I](#)). *EMR1* mRNA expression was not detectable in purified PBMCs, monocytes, or neutrophils; CD34⁺ cells isolated from peripheral blood; mast cells generated by means of *in vitro* differentiation of CD34⁺ cells (data not shown); or any of the human cell lines tested ([Table I](#)).

EMR1 is expressed late in eosinophil development

EMR1 surface expression was examined on CD34⁺ cells in bone marrow aspirates from 9 eosinophilic and 2 healthy donors (representative staining shown in [Fig 2, A](#)) and on purified CD34⁺ cells isolated from peripheral blood of 4 healthy donors (representative staining shown in [Fig 2, B](#)). Although a small population (1% to 4%) of IL-5R α ⁺CD34⁺ cells was identified, EMR1 expression was not detected on CD34⁺ cells in any of the samples studied ([Fig 2, A and B](#)). Purified peripheral blood CD34⁺ cells from 2 donors were differentiated *in vitro* under conditions known to promote eosinophil development to assess the kinetics of EMR1 expression during eosinophilopoiesis. By day 12 after the addition of IL-5, surface expression of CD34 was no longer detected by using flow cytometry ([Fig 2, C](#)), and approximately 20% of the cells were mature eosinophils (Fast Green-positive; [Fig 2, D](#)). As previously described,^{14,15} expression of mRNA for eosinophil granule proteins and *IL-5RA* increased during the culture period, beginning approximately 3 to 4 days after addition of IL-5 and peaking at 10 days ([Fig 2, F and G](#)). Surface expression of EMR1 increased steadily, beginning at approximately 8 days after addition of IL-5 ([Fig 2, E](#), and see [Fig E1, B](#), in this article's Online Repository at www.jacionline.org), and double-staining confirmed that EMR1⁺ cells were also positive for IL-5R α and Siglec-8, which is consistent with an eosinophil phenotype (see [Fig E1, C](#)). *EMR1* mRNA expression was first detected at day 4 and slowly increased throughout the culture period ([Fig 2, G](#)).

EMR1 is expressed on tissue eosinophils

Because the pathogenesis of eosinophilic disorders is primarily due to tissue infiltration by eosinophils, EMR1 expression on eosinophils in skin and nasal polyp tissue was examined by using immunostaining. A formalin-fixed skin biopsy specimen from a subject with HES showed scattered eosinophils ([Fig 3, A](#)), with

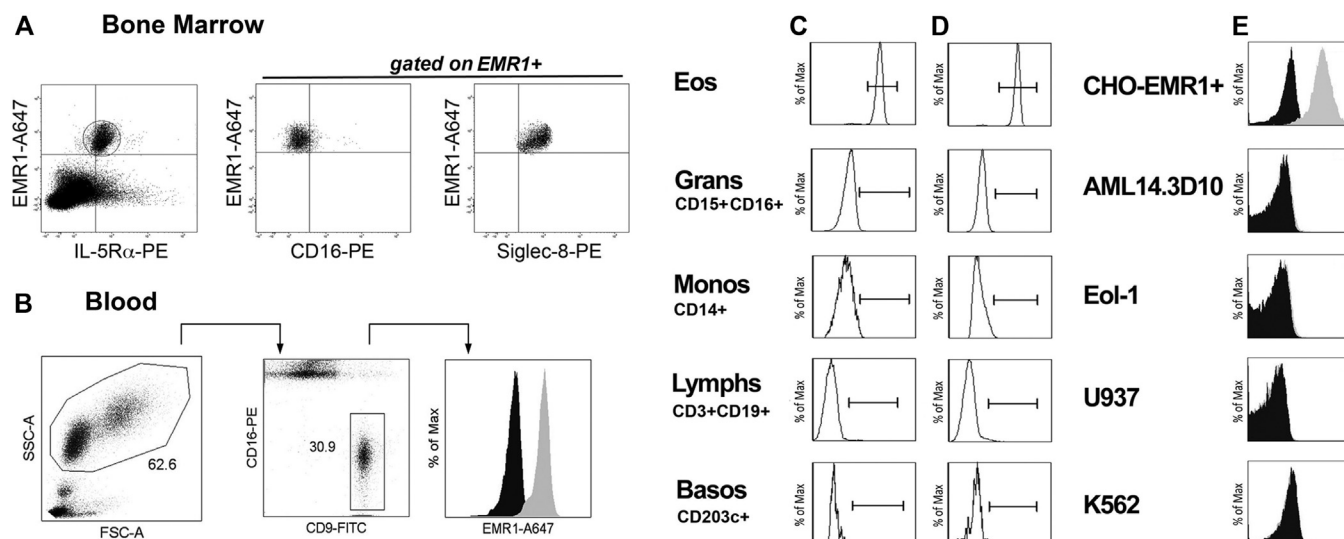


FIG 1. EMR1 is expressed exclusively on eosinophils. **A** and **B**, Representative staining of eosinophils with anti-EMR1 antibody in bone marrow (Fig 1, **A**) and blood (Fig 1, **B**) from a subject with HES. FITC, Fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter. **C-E**, Absence of EMR1 expression on cells other than eosinophils in bone marrow (Fig 1, **C**) and peripheral blood (Fig 1, **D**) and in cell lines (Fig 1, **E**). The CHO-K1SV cell line (CHO cells transfected with EMR1) is shown as a positive control.

TABLE I. EMR1 mRNA expression

Cell type	No.	EMR1 mRNA*
EOS eosinophils	28	19.7 ± 4.6
ND eosinophils	11	8.9 ± 2.5
PBMCs	12	1.6 ± 0.7
Monocytes	4	0.28 ± 0.14
Neutrophils	4	0.06 ± 0.02
CD34 ⁺	4	0.06 ± 0.03
U937	1	0.045
K562	1	0
Eol-1	3	0.08 ± 0.03
AML14.3D10	3	0.20 ± 0.10
CHO-EMR1 ⁺	3	7.97 ± 1.98

EOS, Eosinophilic donor; ND, healthy donor.

*In arbitrary units ($\times 10^{-5}$) related to 18S rRNA (mean $\pm$ SE).

evidence of degranulation demonstrated on immunostaining with anti-EPX antibody (Fig 3, **C**). Anti-EMR1 antibody staining of an adjacent frozen section identified scattered cells in a pattern consistent with intact eosinophils (Fig 3, **B**). Immunofluorescence staining of nasal polyp tissue showed colocalization of cells staining positively with anti-EMR1 with EPX⁺ eosinophils (Fig 3, **D-F**) but not CD68⁺ macrophages (Fig 3, **G-I**).

EMR1 expression is correlated with eosinophilia

Eosinophil surface expression of EMR1 was quantified in whole blood from healthy donors ($n = 16$), eosinophilic subjects receiving no treatment ($n = 14$), and treated subjects with persistent eosinophilia ($n = 7$). Although EMR1 was highly expressed on eosinophils from all subjects, the GM change in mean fluorescence intensity was significantly increased in the healthy donors (5645; 95% CI, 4006-7956) compared with that seen in either the untreated (2832; 95% CI, 1786-4489) or treated (2393; 95% CI, 1248-4587) subjects with eosinophilia ($P < .05$;

Fig 4, **A**). Furthermore, AEC was negatively correlated with surface expression of EMR1 ($r = -0.46$, $P < .001$; Fig 4, **B**), suggesting that modulation of EMR1 expression occurs *in vivo*. To further explore this possibility, surface expression of EMR1 and CD69, an early activation marker, was assessed *in vitro* on purified eosinophils from 8 eosinophilic subjects and 6 healthy donors after incubation for 2 hours in the presence or absence of IL-5 (1 ng/mL). Whereas CD69 expression was upregulated in response to IL-5 in all subjects, expression of EMR1 decreased in 6 of 6 eosinophilic and 4 of 6 healthy subjects, and GM change in mean fluorescence intensity for EMR1 showed a significant, although small, decrease from 3845 to 3475 ($P < .05$; see Fig E2, **A** and **B**, in this article's Online Repository at www.jacionline.org).

Levels of sEMR1 were assessed by using ELISA in undiluted plasma from 13 eosinophilic and 9 healthy control subjects. sEMR1 was detectable in 15 (68%) of the subjects tested, and levels were positively correlated with AECs ($r = 0.69$, $P < .001$; Fig 1, **C**). sEMR1 was not detected in culture supernatants of IL-5-activated eosinophils ($n = 5$, data not shown).

EMR1 mRNA levels were positively correlated with AECs ($r = 0.36$, $n = 39$, $P < .05$; Fig 4, **D**) and were significantly increased in eosinophilic donors compared with healthy donors ($P < .05$, data not shown). *In vitro* stimulation of purified eosinophils with IL-5 at 1 ng/mL overnight or for 6 hours also induced upregulation of EMR1 transcription, with GM mRNA levels increasing from 4.4 to 9.7 arbitrary units ($\times 10^{-5}$) related to 18S rRNA ($n = 13$, $P < .01$; see Fig E2, **C**).

Eosinophil killing is enhanced in the presence of afucosylated anti-EMR1 antibody

Human IgG₁ lacking the monosaccharide fucose in its carbohydrate moiety has been shown to have enhanced ADCC activity because of increased binding affinity for human FcγRIIIa

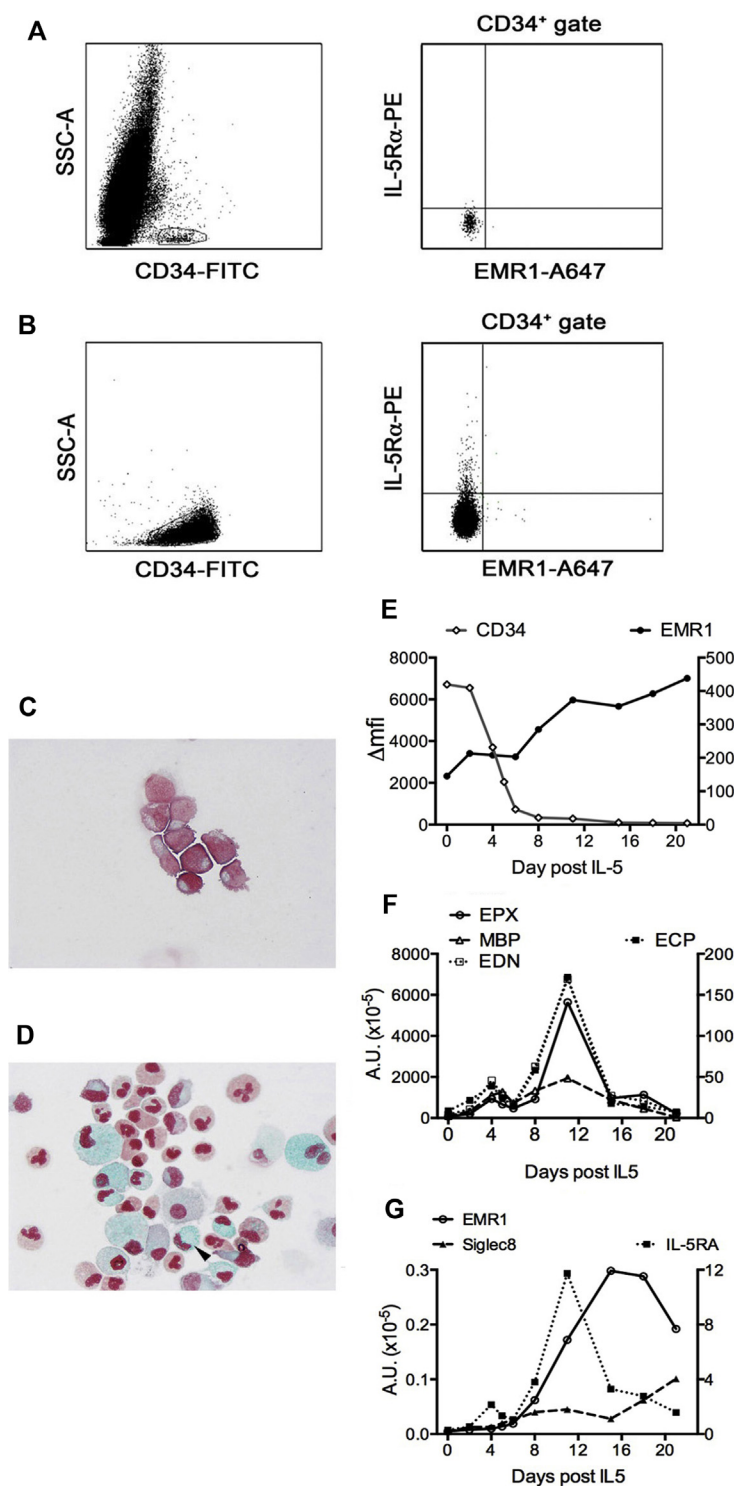


FIG 2. EMR1 is expressed late in eosinophil development. **A** and **B**, Surface expression of IL-5Rα, but not EMR1, is detected on CD34⁺ cells in bone marrow from a patient with HES (Fig 2, **A**) and on purified CD34⁺ cells isolated from peripheral blood (Fig 2, **B**). FITC, Fluorescein isothiocyanate; PE, phycoerythrin; SSC, side scatter. **C** and **D**, *In vitro* differentiation of CD34⁺ cells. Fast Green/Neutral Red staining of cultured CD34⁺ cells 2 days before (Fig 2, **C**) and 21 days after (Fig 2, **D**) the addition of IL-5 is shown. The arrow indicates a mature eosinophil with bilobed red nucleus and green secondary granules. **E**, Surface expression of EMR1 and CD34 as a function of time. Δmfi, Change in mean fluorescence intensity. **F** and **G**, mRNA expression as a function of time for EPX, MBP, and EDN (Fig 2, **F**, left axis); ECP (Fig 2, **F**, right axis); EMR1 and Siglec-8 (Fig 2, **G**, left axis); and IL-5RA (Fig 2, **G**, right axis).

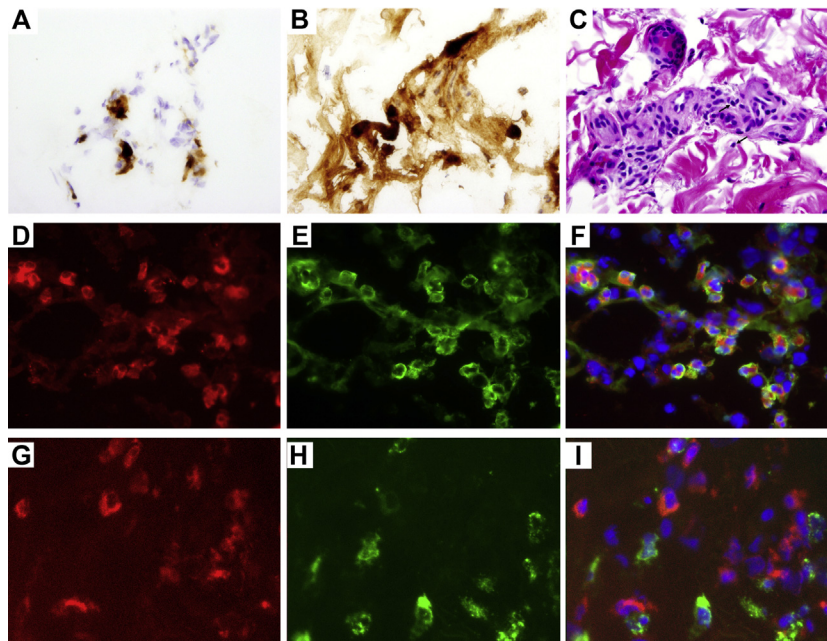


FIG 3. EMR1 expression on tissue eosinophils. **A-C**, Skin biopsy specimen from a subject with HES. Frozen section stained with antibody to EMR1 (Fig 3, A) is shown. Adjacent paraffin-embedded sections stained with antibody to EPX (Fig 3, B) and hematoxylin and eosin (Fig 3, C) showing eosinophilic infiltration and degranulation are also presented. The *arrows* indicate representative eosinophils (Fig 3, C). Fig 3, A and B, Original magnification $\times 600$ and $\times 100$ (Fig 3, C). **D-I**, Nasal polyp biopsy specimen. Immunofluorescence staining of EMR1 in red (Fig 3, D and G), EPX (Fig 3, E) or CD68 (Fig 3, H) in green, and an overlay (Fig 3, F and I) showing EMR1 staining on eosinophils and not on macrophages is shown. Original magnification $\times 640$.

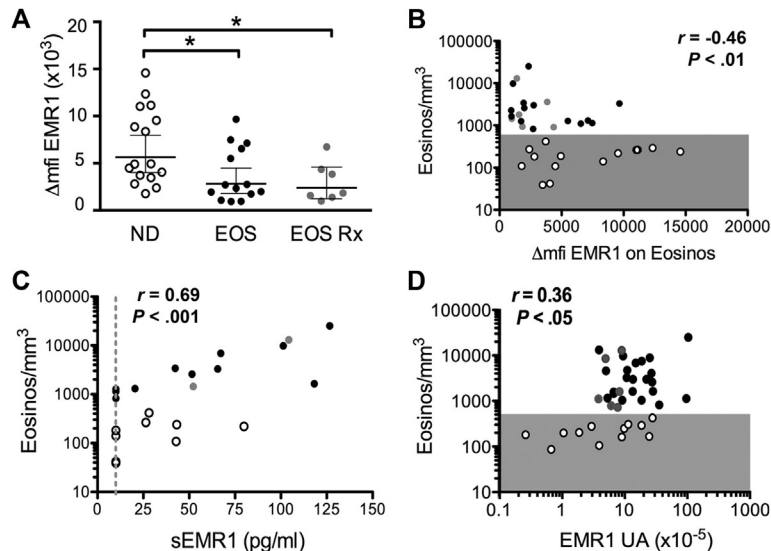


FIG 4. EMR1 expression in eosinophils is correlated with AECs. **A**, Surface expression of EMR1 on eosinophils in whole blood expressed as the change in mean fluorescence intensity (Δmfi) in healthy donors (ND), untreated subjects with eosinophilia (EOS), and treated subjects with eosinophilia (EOS Rx). GMs with 95% CIs are indicated by *horizontal lines*. * $P < .05$ versus healthy donors. **B-D**, Correlation of surface expression of EMR1 (Δmfi), plasma levels of sEMR1 (in picograms per milliliter), and EMR1 mRNA levels (expressed as arbitrary units relative to 18S rRNA) with AECs. An AEC of less than 500/mm³ is indicated by *gray shading*. Each *symbol* represents data for an individual donor. Healthy donors are indicated by *open circles*, eosinophilic subjects receiving no treatment are indicated by *black circles*, and eosinophilic subjects receiving therapy are indicated by *gray circles*.

receptor.¹¹ Consequently, an afucosylated chimeric anti-EMR1 mAb (c1E7) was generated and tested in an *in vitro* NK cell-mediated killing assay by using purified eosinophils as target

cells from 4 healthy and 2 eosinophilic human donors (Fig 5, A, and see Fig E3 in this article's Online Repository at www.jacionline.org). The GM percentage of Annexin V⁺ eosinophils

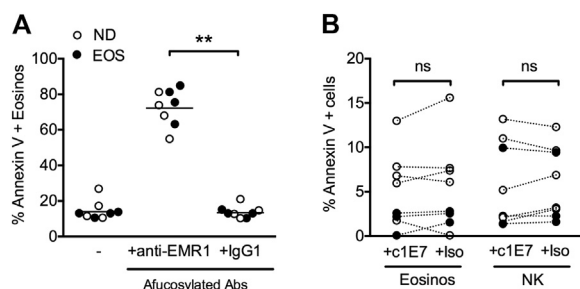


FIG 5. *In vitro* killing of peripheral blood eosinophils with afucosylated anti-EMR1 mAb (c1E7). **A**, Eosinophil (target) cell death after 4 hours of incubation with c1E7 or afucosylated isotype control (*Iso*) and autologous NK cells (effector; effector/target ratio of 5:1). **B**, Lack of eosinophil or NK cell death after 4 hours of incubation with antibody alone. Each symbol represents data for an individual subject. Eosinophilic subjects (*EOS*; $n = 4$) are indicated by black circles, and healthy donors (*ND*; $n = 4$) are indicated by open circles. *CM*, Culture medium; *ns*, not significant. $**P < .01$, Wilcoxon *t* test.

increased from 15.1% to 62.3% in the presence of afucosylated c1E7 ($P < .05$) but not in the presence of antibody isotype control (Fig 5, A) or fucosylated c1E7 antibody at concentrations up to 10 $\mu\text{g/mL}$ (data not shown). Afucosylated c1E7 had no effect on the viability of eosinophils or NK cells when incubated with these cells separately (Fig 5, B).

Eosinophils are depleted *in vivo* after a single dose of afucosylated c1E7 antibody

The chimeric antibody c1E7 binds with similar affinity to recombinant human or cynomolgus macaque EMR1, as determined by using ELISA (data not shown). The antibody was administered intravenously to cynomolgus monkeys at a dose of 1 mg/kg (group 1, $n = 2$) or 5 mg/kg (group 2, $n = 2$). Mean peak plasma concentrations of c1E7 were 41.1 $\mu\text{g/mL}$ for group 1 and 146.7 $\mu\text{g/mL}$ for group 2. The plasma concentration of c1E7 showed a biexponential elimination profile and a terminal phase in all animals, with a mean elimination-phase half-life of 89 hours for group 1 and 210 hours for group 2. Mean volume of distribution of central compartment and volume of distribution at steady state values for each group approximated blood volume consistent with limited tissue distribution (see Table E1 in this article's Online Repository at www.jacionline.org).

Prior studies in cynomolgus monkeys have demonstrated little variability in AECs over time in the absence of treatment.^{16,17} In contrast, peripheral blood eosinophils decreased after infusion in all 4 animals, becoming undetectable at the first time point measured (8 hours) in group 1 and at 2 hours after infusion in group 2 (Fig 6 and see Table E2 in this article's Online Repository at www.jacionline.org). Eosinophil counts returned to normal by day 28 after infusion in the group 1 animals but remained less than baseline counts for 28 to 56 days in the 2 animals that received the 5 mg/kg dose (Fig 6). A mild rebound eosinophilia was noted in 3 of the 4 animals at day 42, resolving by day 56 in the one animal (CYN 10-65c) for which data are available (Fig 6 and see Table E2). The total leukocyte counts excluding eosinophils remained stable after treatment, and no clinically significant adverse events were reported after antibody administration. Plasma levels of eosinophil granule proteins (major basic protein [MBP], eosinophil cationic protein [ECP], eosinophil-derived neurotoxin [EDN], and EPX) were measured at each of the time

points by using a suspension array assay in multiplex (see the Methods section in this article's Online Repository) and showed no consistent trend in the first 24 hours after infusion but decreased in all 4 animals over the 28 days after infusion (see Fig E4 in this article's Online Repository at www.jacionline.org). Additional pharmacokinetic and safety data can be found in Tables E1 and E3 in this article's Online Repository at www.jacionline.org.

DISCUSSION

In their recent report the NIH Taskforce on the Research Needs of Eosinophil-associated Diseases listed the development of new eosinophil-targeted therapeutics as a research priority.¹⁸ In this regard the present study not only confirms previously published data demonstrating that EMR1 expression is restricted to eosinophils⁹ but expands this finding to eosinophils from patients with a wide variety of eosinophilic disorders and provides the first evidence that an afucosylated mAb directed against EMR1 can selectively target and safely eliminate eosinophils *in vivo* in a primate model. Benralizumab (MedImmune, Gaithersburg, Md), an afucosylated antibody against IL-5R α , has already been used in a phase I trial in patients with mild asthma, demonstrating that enhanced ADCC can be used to deplete eosinophils in human subjects.^{17,19} Although no severe adverse events were reported, none of the subjects in this study had marked eosinophilia ($>1500/\mu\text{L}$) at baseline.

A major limitation of targeted therapies currently in clinical trials for eosinophilic disorders has been incomplete depletion of tissue eosinophils. At least in part, this is likely due to alterations in eosinophil surface receptor expression that occur during tissue migration and activation.³ In the present study EMR1 was detected on tissue eosinophils in skin from a patient with HES, nasal polyps, and asthmatic lung disease (data not shown). Of note, EPX staining in the same tissues detected both intact eosinophils and free granules, which is consistent with eosinophil activation. Together these data suggest that EMR1 expression is preserved in activated tissue eosinophils. Nevertheless, the extent to which afucosylated antibody to EMR1 will deplete these tissue eosinophils awaits further study.

EMR1 offers several advantages as a potential therapeutic target. Unlike many eosinophil surface receptors, including IL-5R α , EMR1 is not expressed on mast cells or basophils, precluding unanticipated adverse effects caused by mediator release during cytolysis of these cells. Similar to Siglec-8,²⁰ EMR1 is expressed relatively late in eosinophil development and is unlikely to deplete CD34⁺ eosinophil precursors, which play a potentially important role in plasma cell homeostasis.^{21,22} Finally, although the function of EMR1 is unknown, cynomolgus monkeys experienced no clinically significant adverse effects after administration of anti-EMR1 antibody, and mice deficient in F4/80 (the murine homolog of EMR1) are healthy and fertile.²³

Modulation of cell-surface receptors can occur through a number of different mechanisms, including receptor internalization, proteolytic cleavage, and decreased transcription of mRNA for the membrane form of the receptor. The observed correlation of the peripheral eosinophil count with decreased surface expression of EMR1 on eosinophils and increased plasma levels of sEMR1 supports the hypothesis that EMR1 is shed *in vivo* in response to signals that promote eosinophilia. The fact that soluble receptor was not detected in culture supernatants despite

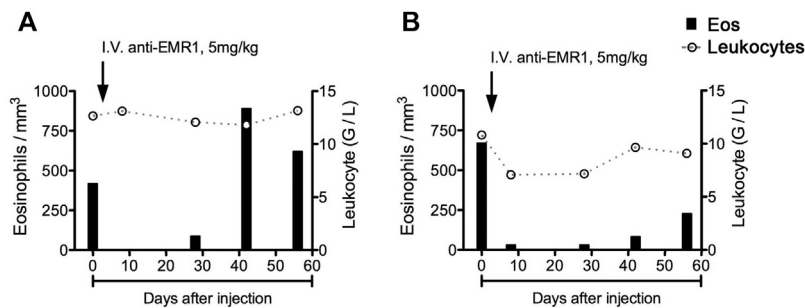


FIG 6. Eosinophil depletion over time after intravenous administration of a single 5 mg/kg dose of afucosylated anti-EMR1 mAb in 2 cynomolgus monkeys. Each panel shows the data for an individual monkey (CYNO 10-65c in **A** and CYNO 10-66c in **B**). The AEC (left axis) at each time point is represented by dark columns, and the white blood cell count (right axis) is indicated by open circles connected by a dashed gray line. I.V., Intravenous.

downmodulation of surface receptor by IL-5 is most likely due to technical constraints related to the concentration of eosinophils in culture, the small change in surface expression in response to IL-5, and the sensitivity of the EMR1 ELISA. Similar findings have been reported for the EGF-7-transmembrane family member CD97, in which increased expression of CD97 in the synovium was accompanied by detectable soluble CD97 in the synovial fluid in subjects with rheumatoid arthritis but not in normal serum or activated T-cell supernatants.^{24,25} Although the mechanism of receptor shedding was not addressed in this study, autoproteolysis of CD97 and EMR2 has been demonstrated to occur through G protein-coupled receptor proteolytic sites in their extracellular domains.²⁶ EMR1 also contains a G protein-coupled receptor proteolytic site; however, the sequence is imperfect, and functionality of the EMR1 domain has not been demonstrated to date.²⁷

Although decreased surface receptor and increased soluble receptor levels could both theoretically interfere with the efficacy of a therapeutic antibody to EMR1 in patients with marked eosinophilia, this is unlikely for the following reasons. First, despite the small but significant decrease with increasing AECs, eosinophil surface expression of EMR1 remained high in all subjects tested. Second, *in vitro* killing was equally efficient in subjects with and without eosinophilia. Third, plasma levels of soluble receptor (10-126 pg/mL; equivalent to approximately 0.1-1.5 pmol/L) were several logs lower than the levels of chimeric afucosylated antibody (2.15 µg/mL [or 14.3 nmol/L] after 15 days and 11.1 µg/mL [or 74 nmol/L] after 28 days for doses at 1 and 5 mg/kg, respectively) achieved in pharmacokinetic studies in nonhuman primates (see Table E3).

In summary, EMR1 is expressed exclusively on mature eosinophils in the blood, bone marrow, and tissues of patients with eosinophilia, regardless of the underlying disorder. Although receptor expression is decreased on peripheral blood eosinophils in the setting of eosinophilia, IL-5 exposure, or both, this downmodulation was not sufficient to impair *in vitro* killing by NK cells in the presence of afucosylated anti-EMR1 antibody. More importantly, administration of a single dose of afucosylated chimeric anti-EMR1 antibody induced rapid and sustained depletion of peripheral blood eosinophils without effect on other peripheral blood cells or evidence of clinical toxicity. Although the number of animals studied was small, these findings suggest that selectively targeting eosinophils using a Humanized afucosylated anti-EMR1 antibody might provide a safe and effective strategy for the treatment of eosinophilic disorders.

We thank Nicole Holland-Thomas, Kathryn Spates, and Amara Pabon for their assistance in procuring the subject samples for these studies. We thank Swathi Sujatha-Bhaskar for characterization of EMR1 antibodies; Jason Williams, Wendy Ching, and Thomas Chang for EMR1 production and characterization; and Kenneth Luehrsens and David Martinez for generating EMR1 DNA constructs.

Clinical implications: Afucosylated antibody to the human eosinophil-specific surface receptor EMR1 shows promise as a new therapy for eosinophilic disorders.

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METHODS

Detection of surface EMR1 by means of flow cytometry

EMR1 expression in bone marrow aspirates and peripheral blood was assessed by means of multiparameter flow cytometry with directly conjugated antibodies against CD45 (clone 2D1), CD34 (clone 8G12), IL-5R α (CDw125; clone A14), CD16 (clone 3G8), CD9 (M-L13), CD14 (M5E2), CD49d (clone 9F10), CD117 (clone 104D2), CD19 (SJ25C1), CD15 (HI98), CD3 (SK7), CD69 (FN50) (BD Biosciences), CD203 (97A6) (Beckman Coulter, Indianapolis, Ind), CD10 (MEM78) and CD64 (10.1) (Invitrogen), Siglec-8 (7C9; BioLegend, San Diego, Calif), and anti-EMR1 (clone 1E7; Kalobios Pharmaceuticals, South San Francisco, Calif). After antibody staining, red blood cells were lysed and samples were fixed, as previously described.^{E1} Eosinophils were identified in whole blood as CD45⁺CD16⁺CD9⁺ cells with characteristic high side-scatter properties after doublet exclusion. Irrelevant directly conjugated mouse IgG₁ was used to ascertain background staining, and antibodies were titrated before use. Whole blood and bone marrow aspirates were analyzed on a FACSCanto II flow cytometer (BD Biosciences) with FCS Express 4 software (De Novo Software, Los Angeles, Calif) for data analysis. Cell lines and purified cells were analyzed on an LSR II flow cytometer by using Diva software (BD Biosciences). FlowJo software (version 9.5.2; Tree Star, Ashland, Ore) was used for data analysis.

Primers used for real-time quantitative PCR

EMR1 and *Siglec-8* mRNA were amplified by using previously published primer sets^{E2,E3} and 1X SYBR Green (SA Biosciences, Frederick, Md) in a final 20- μ L reaction volume. Forward and reverse primer sequences for the 18S control were 3'-GCCCGAAGCGTTACTTTGA-5' and 3'-TCCAT TATTCCTAGCTGCGGTATC-5', respectively. The cationic proteins *MBP*, *EPX*, *ECP*, *EDN*, and *IL5RA* (membrane form) were amplified by using the following commercially available TaqMan primers (Applied Biosystems) in a final volume of 10 μ L, according to the manufacturer's instructions: *MBP*, *PRG2*, Hs00794928_m1; *EPX*, Hs00946094_m1; *ECP*, *RNAse2*, Hs00795553_s1; *EDN*, *RNAse3*, Hs00795553_s1; *IL-5RA*, s00602482_m1; and *18S*, X03205.1.

Immunostaining

Immunohistochemical staining for EPX was performed on formalin-fixed paraffin sections of a skin biopsy specimen by using a mouse anti-mouse mAb (1:500, provided by Dr J. J. Lee), as previously described.^{E4} Slides were deparaffinized, and heat-induced epitope retrieval was accomplished with Leica retrieval solution (citric buffer) for 25 minutes. Immunostaining for EMR1 was performed on an adjacent frozen sample of the same biopsy specimen with mouse 1E7 antibody at 10 μ g/mL. Slides were prepared by Histoserv (Germantown, Md). Anti-EPX and anti-EMR1 antibodies were detected with the BondMax biotin-avidin free polymer-based detection system, with diaminobenzidine as the chromogen. Samples were analyzed on a Leica BondMax autostainer (Leica Microsystems, Bannockburn, Ill).

Immunofluorescence staining for EMR1, EPX, and CD68 was performed on acetone-fixed nasal polyp frozen sections. Slides were washed twice with PBS with 0.1% Tween 20 (PBST) before incubation with 700 μ L of blocking solution (2% BSA, 1.5% goat serum [Abcam], and 1.5% donkey serum [Abcam] in PBS) at room temperature for 1 hour. After 3 washes with PBS, slides were incubated overnight at 4°C with mouse 1E7–Alexa Fluor 595 (10 μ g/mL), mouse anti-EPX antibody (diluted 1:200 with 2% BSA with PBS, Abcam), and/or mouse anti-CD68 (clone KP-1, diluted 1:2; Ventana). The slides treated with anti-EMR1, anti-EPX, and anti-CD68 were then washed and incubated with goat anti-mouse IgG–DyLight 488 (Jackson ImmunoResearch, West Grove, Pa) at 3 μ g/mL in 2% BSA with PBS at room temperature for 1 hour. After 3 washes with PBST, 2 to 3 drops of mounting

medium (VECTASHIELD Mounting Medium; Vector Laboratories, Burlingame, Calif) were added before cover slipping. Images were taken on a Nikon fluorescent microscope (Nikon, Tokyo, Japan).

c1E7 ELISA

Plasma samples were analyzed for c1E7 by using an antigen-based ELISA. Briefly, a microplate was coated with 100 ng per well of EMR1-Fc (KaloBios Pharmaceuticals) in coating buffer (20 mmol/L Tris and 150 mmol/L NaCl, pH 8.0). After incubation for 1 hour at 37°C with shaking, the plate was washed 3 times with wash buffer (PBST) and blocked for 20 minutes at 37°C with Superblock (Pierce, Rockford, Ill). Diluted cynomolgus monkey plasma samples (1:300 and/or 1:600 for group 1 and 1:1500 and/or 1:3000 for group 2) and recombinant standards were incubated for 1 hour at room temperature. The plate was washed 3 times with wash buffer before addition of peroxidase-conjugated AffiniPure goat anti-human IgG Fab-specific antibody (10 ng/mL in PBST with 1% BSA, Jackson ImmunoResearch). After a final wash step, TMB substrate solution was added (Invitrogen), and the reaction was allowed to proceed for 7 minutes before the addition of stop solution (1N H₂SO₄). Absorbance was read at 450 nm with a Thermomax Molecular Device microplate reader. Data were plotted and fitted by using a 4-parameter logistic regression with SoftMax Pro 4.0 software (Molecular Devices, Sunnyvale, Calif). The concentration in plasma samples was determined based on the values from the standard curve.

Measurement of eosinophil granule proteins

Levels of eosinophil granule proteins (MBP, ECP, EDN, and EPO) were measured in supernatants and primate plasma by using a customized suspension array assay in multiplex (Makiya et al, unpublished data). Coupling of antibodies to the microspheres and assay conditions were performed according to the manufacturer's instructions (Luminex, Austin, Tex). Briefly, supernatants and plasma were reduced and alkylated to remove MBP from complexes^{E5} before incubation at a final dilution of 1:8.8 (supernatants) or 1:44 (plasma) with magnetic microspheres coupled to mouse anti-MBP, anti-ECP, anti-EDN, and anti-EPO mAbs.^{E6} The microspheres were subsequently incubated with rabbit anti-MBP, anti-ECP, anti-EDN, and anti-EPO^{E6} and biotinylated goat anti-rabbit IgG and PE-streptavidin (Jackson ImmunoResearch) and analyzed with a Bio-Plex 200 (Bio-Rad Laboratories, Hercules, Calif) instrument. All assays were performed in duplicate, and concentrations were calculated based on a standard curve with SoftMax Pro Software (Molecular Devices).

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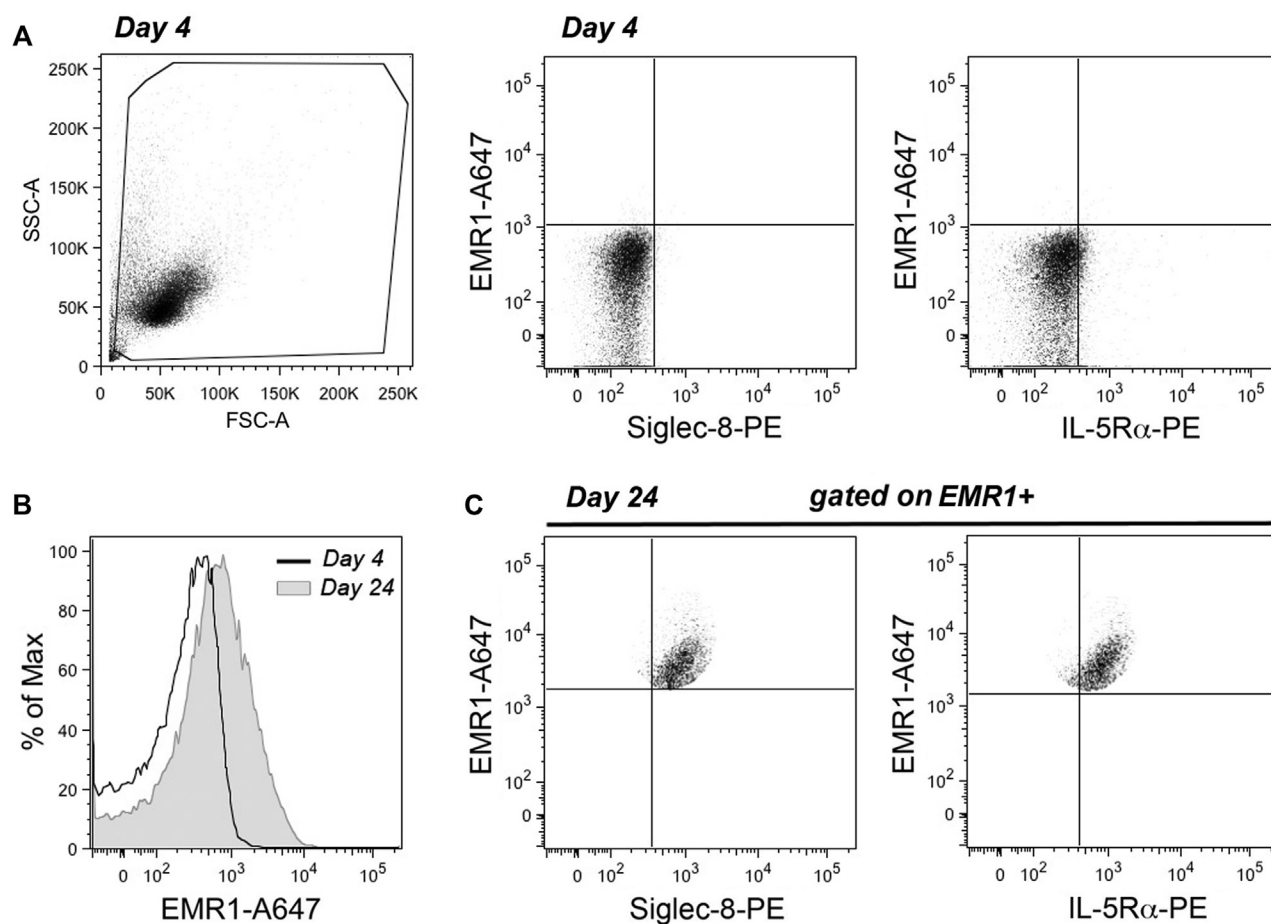


FIG E1. *In vitro* differentiation of CD34⁺ cells. **A**, Representative dot plots of EMR1/Siglec-8 and EMR1/IL-5R α surface expression at day 4. **B**, Overlay of EMR1 staining at days 4 and 24. **C**, Representative dot plots of Siglec-8 and IL-5R α surface expression on EMR1⁺ cells at day 24. *FSC*, Forward scatter; *PE*, phycoerythrin; *SSC*, side scatter.

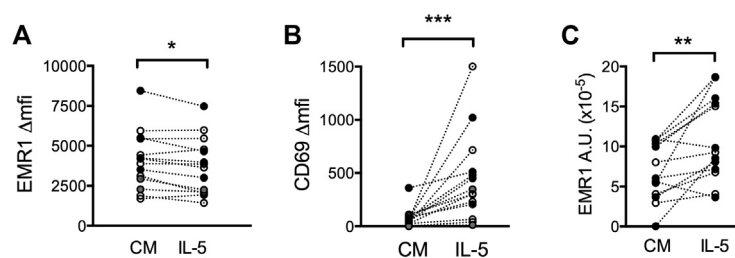


FIG E2. *In vitro* modulation of EMR1 in response to IL-5 stimulation. **A** and **B**, Surface expression of EMR1 and CD69 on purified eosinophils after 2 hours of stimulation ($n = 14$, 6 healthy donors and 8 patients with HES). **C**, *EMR1* mRNA upregulation by IL-5 after 6 hours of stimulation ($n = 13$, 4 healthy donors and 9 patients with HES). *CM*, Culture medium; Δmfi , change in mean fluorescence intensity. * $P < .05$, ** $P < .01$, and *** $P < .001$.

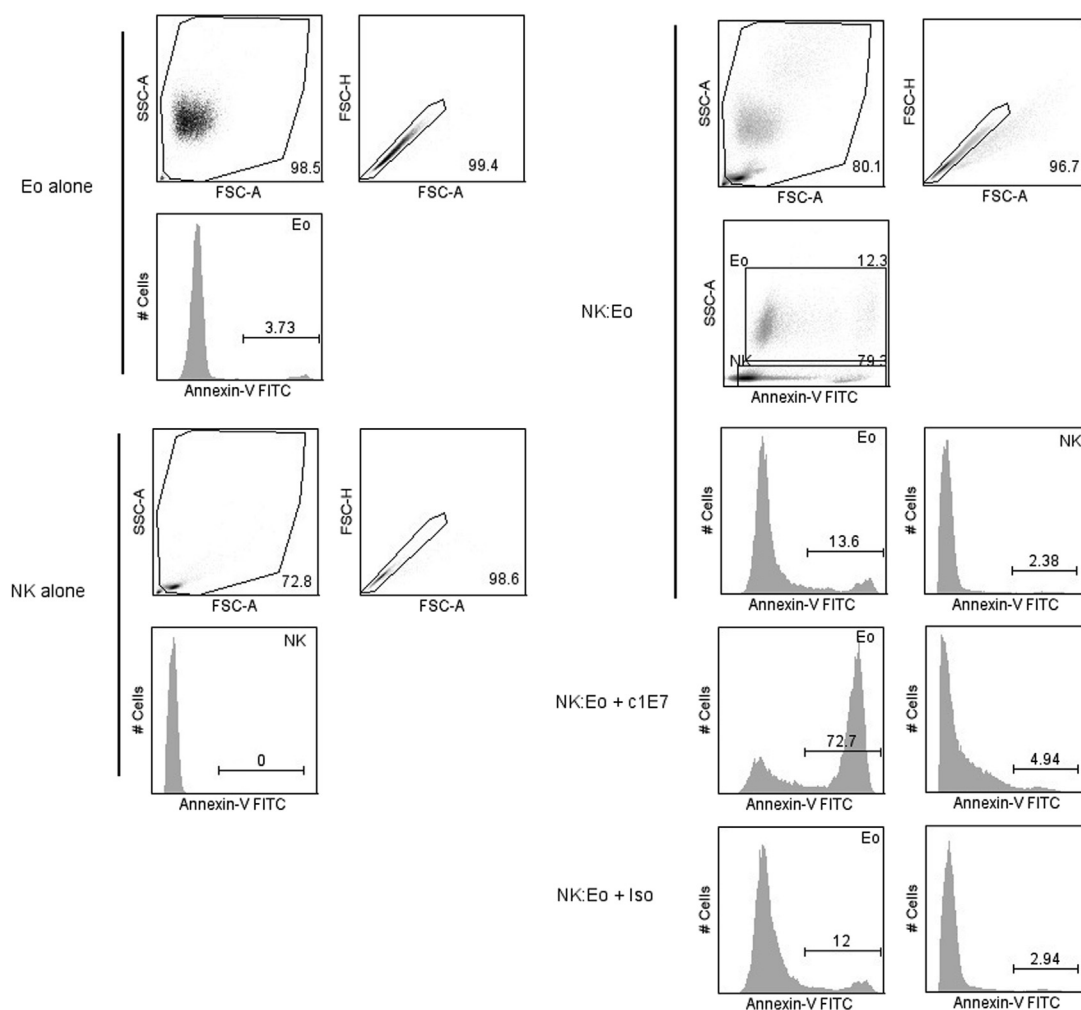


FIG E3. Cytofluorimetric gating for the determination of eosinophil cell death induced by NK cells in the presence of afucosylated anti-EMR1 antibody (c1E7). Results shown are from 1 representative experiment. NK cell-mediated killing assays were performed with purified eosinophils at an effector/target ratio of 5:1. The cells were incubated together for 4 hours and washed before addition of Annexin V-FITC. After exclusion of cell debris and doublets, the percentage of Annexin V⁺ cells was determined in the eosinophil (Eo) and effector NK (NK) cell gates. FITC, Fluorescein isothiocyanate; FSC, forward scatter; SSC, side scatter.

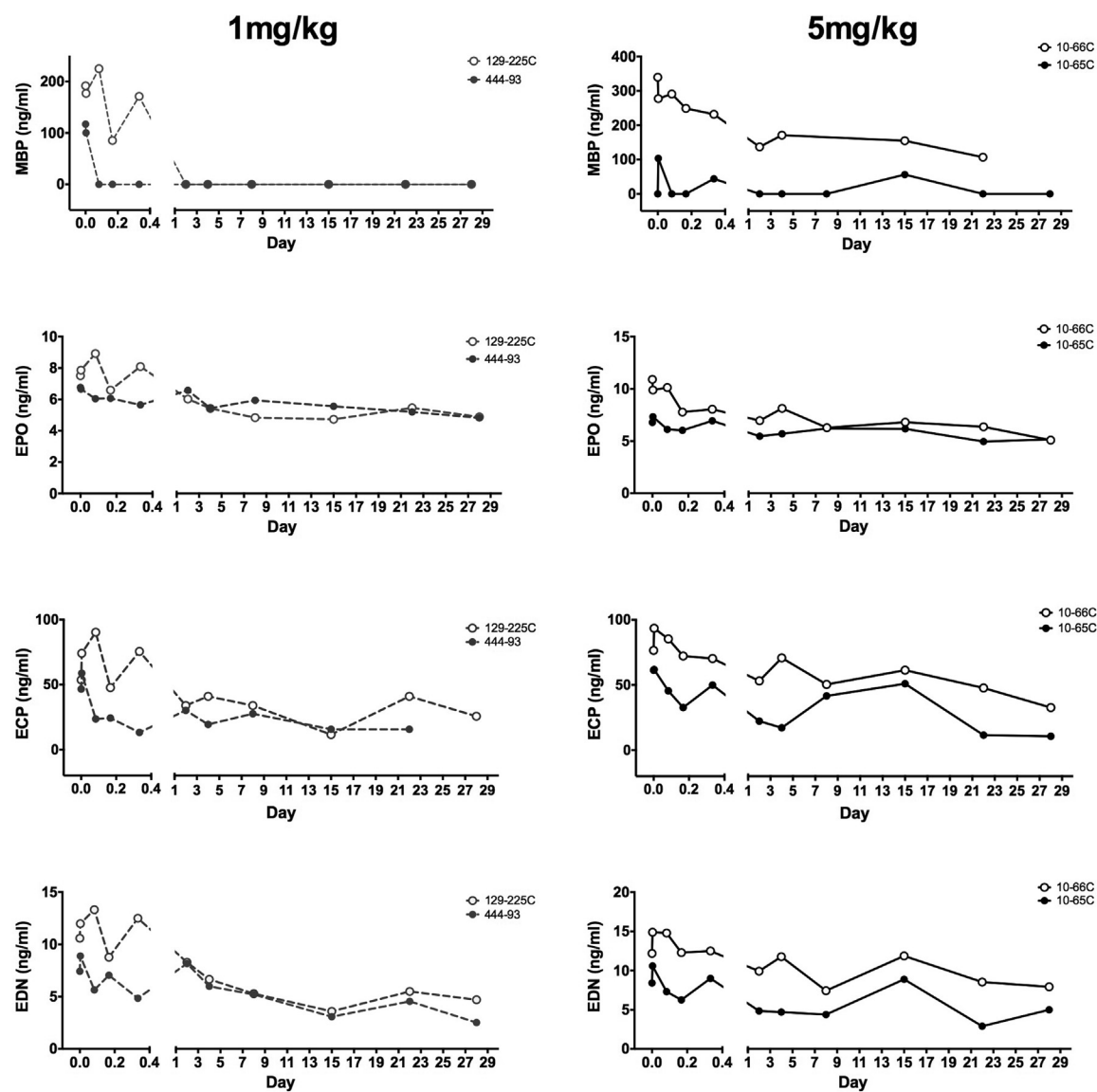


FIG E4. Levels of eosinophil granule proteins (ECP, MBP, EPO, and EDN) in plasma samples from the 4 monkeys at all time points.

TABLE E1. Pharmacokinetic parameters after administration of chimeric afucosylated anti-EMR1 antibody (c1E7) to nonhuman primates

	Dose: 1 mg/kg		Dose: 5 mg/kg	
	CYNO 129-225c	CYNO 444-93	CYNO 10-65c	CYNO 10-66c
C_{max} (μg/mL)	33.3	48.8	130.5	162.8
AUC_{∞} (μg*h/mL)	3,226	2,885	22,132	27,545
CL (mL/kg/h)	310	347	226	182
V_c (mL/kg)	34.2	24.9	37.5	33.8
V_{ss} (mL/kg)	42.7	37.0	68.7	58.4
$t_{1/2}$ (h)	92.2	77.5	205	215

AUC_{∞} , Area under the curve from time 0 extrapolated to infinite time; CL , systemic clearance; C_{max} , maximum concentration; $t_{1/2}$, elimination half-life; V_c , volume of distribution of central compartment; V_{ss} , volume of distribution at steady state.

TABLE E2. Eosinophil counts measured at each time point in the 4 monkeys before and after receiving a single dose of afucosylated anti-EMR1 antibody

Group	Monkey	Time point	Eosinophils (%) [*]	White blood cell count (10 ⁹ /L)	Absolute eosinophil count (/mm ³)
1 (1MPK) CYN 444-93		Predose	5.45	7.93	431.9
		5 min	NA	NA	NA
		2 h	NA	NA	NA
		4 h	NA	NA	NA
		8 h	0.56	NA	NA
		Day 2	0.27	NA	NA
		Day 4	0.30	NA	NA
		Day 8	0.42	NA	NA
		Day 15	1.00	NA	NA
		Day 22	3.98	NA	NA
		Day 28	9.60	NA	NA
		Day 42	11.23	NA	NA
		Day 56	NA	NA	NA
	CYN 129-225c	Predose	7.18	13.80	990.3
		5 min	NA	NA	NA
		2 h	NA	NA	NA
		4 h	NA	NA	NA
		8 h	0.00	NA	NA
		Day 2	0.00	NA	NA
		Day 4	0.00	NA	NA
		Day 8	0.42	NA	NA
		Day 15	0.55	NA	NA
		Day 22	2.95	NA	NA
		Day 28	8.96	NA	NA
		Day 42	9.43	NA	NA
		Day 56	NA	NA	NA
2 (5MPK) CYN 10-65c		Predose	3.32	12.66	420.1
		Predose	5.45	7.90	430.6
		5 min	3.71	NA	NA
		2 h	0.29	NA	NA
		4 h	0.54	NA	NA
		8 h	0.00	NA	NA
		Day 2	0.00	NA	NA
		Day 4	0.32	NA	NA
		Day 8	0.00	13.11	0.0
		Day 15	0.92	NA	NA
		Day 22	0.60	NA	NA
		Day 28	0.74	12.07	88.8
		Day 42	7.55	11.81	891.3
		Day 56	4.72	13.16	621.3
	CYN 10-66c	Predose	6.21	10.82	672.4
		Predose	3.95	8.45	333.8
		5 min	4.10	NA	NA
		2 h	0.00	NA	NA
		4 h	0.00	NA	NA
		8 h	0.31	NA	NA
		Day 2	0.00	NA	NA
		Day 4	0.00	NA	NA
		Day 8	0.48	7.07	33.7
		Day 15	0.96	NA	NA
		Day 22	1.26	NA	NA
		Day 28	0.47	7.17	33.8
		Day 42	0.88	9.65	84.6
		Day 56	2.53	9.09	229.9

NA, Not available.

^{*}Percentage based on cytopsin counting on 200 to 500 cells per slide.

TABLE E3. Laboratory parameters before and after administration of a single 5 mg/kg intravenous dose of chimeric afucosylated anti-EMR1 antibody to 2 cynomolgus monkeys

Laboratory parameter	Normal value	Unit	Predose		Day 8		Day 28		Day 29		Day 42		Day 56	
			CYNO 10-65c	CYNO 10-66c	CYNO 10-65c	CYNO 10-66c	CYNO 10-65c	CYNO 10-66c	CYNO 10-65c	CYNO 10-66c	CYNO 10-65c	CYNO 10-66c	CYNO 10-65c	CYNO 10-66c
Hematology														
White blood cell count	5.7-21	10 ⁹ /L	7.9	10.82	13.11	7.07	12.07	7.17	ND	ND	11.81	9.65	13.16	9.09
Absolute lymphocyte count	4.3-10	10 ⁹ /L	4.65	5.69	6.5	5.05	6.79	5.24	ND	ND	5.59	4.38	6.79	5.49
Absolute monocyte count	0-1	10 ⁹ /L	0.05	0.47	0.4	0.13	0.52	0.04	ND	ND	0.39	0.24	0.33	0.3
Absolute neutrophil count	1.9-6.24	10 ⁹ /L	3.21	4.66	6.21	1.9	4.76	1.9	ND	ND	5.84	5.04	6.04	3.3
Red blood cell count	4.8-6.3	10 ¹² /L	5.79	6.26	5.74	4.93	5.92	5.71	ND	ND	5.64	5.21	5.73	5.74
Platelets	130-934	10 ⁹ /L	334	438	507	378	525	394	ND	ND	461	376	422	336
Hemoglobin	8-15	g/dL	13.2	12.6	11.8	12.6	12.6	12.5	ND	ND	12	12.3	12	13
Clinical chemistry														
Total protein	5.9-7.6	g/dL	7.1	7.2	7.2	7	7.4	7	7	7	7.3	7.2	6.6	6.9
Albumin	2.8-4.4	g/dL	3.5	3.9	3.5	3.9	3.8	3.8	3.8	4	4.1	4.4	3.4	3.8
Globulin	2-3.9	g/dL	3.6	3.3	3.7	3.1	3.5	3.2	3.2	3	3.2	2.8	3.2	3.1
Alkaline phosphatase	73- 210	U/L	353*	298*	347*	345*	292*	273*	305*	258*	361*	286*	411*	386*
Alanine aminotransferase	20-120	U/L	40	45	27	35	VNR	VNR	30	55	28	24	57	41
Amylase	149-500	U/L	156	232	146*	231	171	255	178	273	184	284	149	273
Aspartate aminotransferase	23-94	U/L	17*	36	8*	18*	VNR	VNR	48	22*	36	21*	24	11*
Urea	7-25	mg/dL	16	13	16	14	19	15	21	18	16	12	19	14
Calcium	8.3-10.1	mg/dL	9.1	10.2*	9.6	9.6	9.9	10.4*	10.2*	10.5*	10.2*	10.1	9.7	10.2*
Cholesterol	73-179	mg/dL	173	175	182*	199*	186*	180*	185*	186*	165	185*	162	178
Creatinine	0.4-1.2	mg/dL	0.8	0.7	0.7	0.7	0.8	0.6	0.8	0.6	0.7	0.7	0.7	0.6
Glucose	50-100	mg/dL	83	87	74	98	86	79	78	71	99	103*	73	82*
Inorganic phosphate	2.4-6.5	mg/dL	5.1	4.1	5.5	6.6*	4.3	3.8	5	3.6	4.1	2.5	4.7	4.4
Total bilirubin	0.1-0.6	mg/dL	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

ND, Not done; VNR, value not returned because of sample or machine error.

*Values greater than or less than the normal range.