HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2020 June 01.

Published in final edited form as:

J Allergy Clin Immunol. 2019 June ; 143(6): 2227–2237.e10. doi:10.1016/j.jaci.2018.10.066.

Siglec-8 in eosinophilic disorders: receptor expression and targeting using chimeric antibodies

Fanny Legrand, PhD, PharmD[1], **Yun Cao, MS**[2], **Joshua Wechsler, MD**[3], **Xiang Zhu, PhD**[4], **Nives Zimmermann, MD**[4], **Shakuntala Rampertaap, MT/ASCP**[5], **Joseph Monsale, MT/ASCP**[5], **Kimberly Romito, MT/ASCP**[5], **Bradford A. Youngblood, PhD**[6], **Emily C. Brock, MSc**[6], **Michelle Makiya, MSc**[1], **Nenad Tomasevic, PhD**[6], **Christopher Bebbington, PhD**[6], **Irina Maric, MD**[7], **Dean D. Metcalfe, MD**[8], **Bruce S. Bochner, MD**[2], and **Amy D. Klion, MD**[1]

[1]Human Eosinophil Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases MD

[2]Division of Allergy and Immunology, Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Ill

[3]Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Ann and Robert H. Lurie Children's Hospital of Chicago, Chicago, Ill

[4]Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

[5]Department of Laboratory Medicine, Warren Magnusson Clinical Center, National Institutes of Health, Bethesda, MD

[6]Allakos, Inc., San Carlos, CA

[7]Hematology Section, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, MD

[8]Mast Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

Abstract

Corresponding Author Amy Klion, MD, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bldg. 4, Room B1-28, Bethesda, MD 20892, aklion@nih.gov, Tel: 301-435-8903. **Alternate corresponding author:** Fanny Legrand, PhD, PharmD, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bldg. 4, Room B1-28, Bethesda, MD 20892, fanny.legrand@nih.gov, Tel: 301-761-5348.

Competing Interests

F.L., Y.C., J.W., X.Z., N.Z., S.R., J.M., K.R., M.M., I.M., D.D.M. and A.D.K. declare no conflicts of interest.

B.S.B. has current consulting and scientific advisory board arrangements with Allakos, Inc., and owns stock in Allakos, Inc. He is a co-inventor on existing Siglec-8-related patents and thus may be entitled to a share of royalties received by Johns Hopkins University on the potential sales of such products. B.S.B. is also a co-founder of Allakos, Inc. which makes him subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University and Northwestern University in accordance with their conflict of interest policies. B.A.Y., E.C.B., N.T. and C.B. are full time employees of Allakos Inc.

Background: Sialic acid–binding immunoglobulin-like lectin (Siglec)-8 is selectively expressed on eosinophils, mast cells and basophils, and, when engaged on eosinophils, can cause cell death.

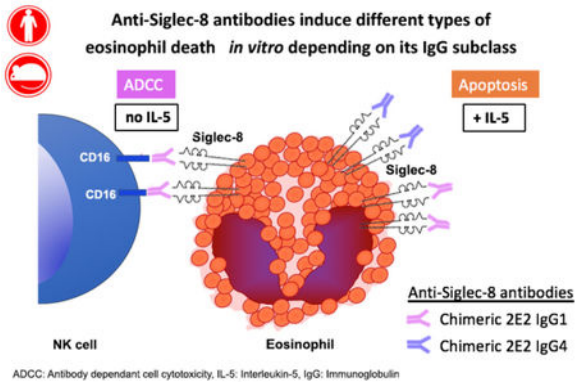
Objective: To characterize surface and soluble Siglec-8 levels in normal (ND) and eosinophilic (EO) subjects and assess the efficacy of anti-Siglec-8 antibodies in inducing eosinophil cell death *in vitro*.

Methods: Eosinophil expression of Siglec-8 was assessed using flow cytometry and quantitative PCR. Serum soluble Siglec-8 levels were measured by ELISA. Induction of eosinophil death by IgG4 (c2E2 IgG4) and afucosylated IgG1 (c2E2 IgG1) anti-Siglec-8 antibodies was evaluated *in vitro* by flow cytometry and *in vivo* in humanized mice.

Results: Siglec-8 was consistently expressed on eosinophils from ND and EO and did not correlate with absolute eosinophil count (AEC) or disease activity. Soluble Siglec-8 levels were measurable in serum from most donors, unrelated to AEC or Siglec-8 surface expression. c2E2 IgG1 and c2E2 IgG4 were equally effective at inducing cell death (Annexin-V positivity) of purified eosinophils from ND and EO after overnight IL-5 priming. In contrast, killing of purified eosinophils without IL-5 was only seen in EO subjects, and NK-mediated eosinophil killing was seen only with c2E2 IgG1. Finally, treatment of humanized mice with anti-Siglec antibody led to robust depletion of IL-5-induced eosinophilia *in vivo*.

Conclusions: Siglec-8 is highly expressed on blood eosinophils from EO and ND and represents a potential therapeutic target for eosinophilic disorders. Enhanced killing of eosinophils in the presence of IL-5 may lead to increased efficacy in patients with IL-5-driven eosinophilia.

Graphical Abstract



Capsule Summary

Siglec-8 levels are similar on blood eosinophils irrespective of eosinophil count or disease activity. Anti-Siglec-8 antibodies induce death of eosinophils from eosinophilic donors *in vitro* and in a humanized mouse model of IL-5-driven eosinophilia.

Keywords

Siglec-8; eosinophil; mast cell; soluble receptor; inhibitory receptor; apoptosis; hypereosinophilic syndrome; eosinophilic gastrointestinal disease; mastocytosis; monoclonal antibody

Introduction

Despite the recent expansion in biologics for eosinophilic disorders, none has proven effective in reducing blood and tissue eosinophilia in all subjects[1]. Additional approaches are clearly needed, especially for the heterogeneous group of disorders that constitute hypereosinophilic syndromes (HES)[2]. Siglec-8 is a surface receptor expressed exclusively on mature eosinophils, mast cells and basophils[3]. Crosslinking of Siglec-8 *in vitro* induces eosinophil death[3], and this process is dependent on ROS release and activation of β2 integrin[4,5]. Priming *in vitro* with cytokines, including IL-5, GM-CSF and IL-33, potentiates cell death and obviates the need for crosslinking with a second antibody[6]. Unlike eosinophils, mast cells do not die in response to crosslinking of Siglec-8; however, FcεRI-mediated secretion is inhibited[7].

Restricted expression of Siglec-8 on effector cells involved in allergic inflammation and the fact that receptor engagement leads to eosinophil cell death makes Siglec-8 an attractive target for therapeutic antibody development. In this study, the activity of two chimeric anti-Siglec-8 antibodies was compared on eosinophils from eosinophilic and normal donors. c2E2 IgG4 is a chimeric IgG4 version of a mouse anti-Siglec-8 antibody shown to induce eosinophil apoptosis *in vitro*[3]. c2E2 IgG1 is a chimeric afucosylated IgG1 antibody with the same specificity as c2E2 IgG4 that is anticipated to enhance antibody-dependent cellular cytotoxicity (ADCC) through NK cell engagement of CD16, as described with benralizumab, an afucosylated IgG1 anti-IL-5 receptor antibody approved for the treatment of asthma[8,9].

Although prior studies in small numbers of normal donors (ND) and patients with HES have demonstrated Siglec-8 expression on peripheral blood eosinophils[10] and detected soluble Siglec-8 (sSiglec-8) in serum from some HES patients[11], little is known about the variability and regulation of Siglec-8 expression *in vivo*. A weak correlation was noted between serum soluble Siglec-8 levels and absolute eosinophil count (AEC)[11]. In the present study, Siglec-8 surface expression and serum levels of sSiglec-8 were charatcerized in a large cohort of normal and eosinophilic donors and the effects of c2E2 IgG4 and c2E2 IgG1 on eosinophil viability and depletion were explored *in vitro* and in a humanized mouse model.

Methods

Anti-Siglec-8 mAbs

Chimeric 2E2 IgG4 (c2E2 IgG4) mAb and chimeric 2E2 IgG1 (c2E2 IgG1) mAb are recombinant antibodies comprised of murine variable regions specific for Siglec-8 attached to human constant regions. Details of their production are in the Supplemental Methods. Preclinical grade fully-human IgG1 and IgG4 antibodies were used as isotype controls (Eureka Therapeutics, CA).

Study subjects

Subjects with eosinophilia (EO) or D816V *KIT*-positive systemic mastocytosis (SM) underwent detailed clinical evaluation at the NIH Clinical Center on NIAID Institutional Review Board (IRB)-approved protocols to study these disorders (NCT00001406 and

NCT00044122). ND were recruited under an NIAID IRB-approved clinical protocol for *in vitro* research (NCT00090662). Subjects with eosinophilic esophagitis (EoE) were part of a prospective cohort followed at Ann & Robert H. Lurie Children's Hospital of Chicago from November 2010 to May 2015 on protocol IRB 2010–14155. EoE was defined as symptoms of esophageal dysfunction of unknown cause and >15 eosinophils per high power field (eos/hpf) on esophageal biopsy after 6–8 weeks of proton pump inhibition. Non-EoE controls had symptoms of esophageal dysfunction, but esophageal biopsies which did not meet the histologic criteria for EoE. All participants gave written informed consent. Demographic and laboratory characteristics of the different patient populations are given in Supplemental Table 1.

Eosinophil and NK cell purification and culture conditions

Peripheral blood was collected for all experiments in tubes containing ETDA anticoagulant. Eosinophils were purified by magnetic bead selection (Miltenyi Biotech, Cambridge, Mass) as previously described [12]. Autologous NK cells were purified by negative selection from peripheral blood mononuclear cells using the NK Cell Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. 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 NK cells was determined by 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, Carlsbad, CA) at a concentration of 10^7/mL. 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 mg/mL gentamicin (culture medium).

Real-time quantitative PCR

Total RNA was extracted from purified eosinophils in TriZol according to the manufacturer's instructions prior to cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Siglec-8 cDNA and 18S rRNA were amplified in a 96-well plate (final volume: 10 μl) using the following commercially available TaqMan primers (Applied Biosystems): Hs00274289_ m1, and X03205.1, respectively. Samples were run in triplicate. mRNA levels are expressed in arbitrary units (x 10^{-5}) related to 18S rRNA (mean ± SE), determined by $10^5 \times 1/(2^{\Delta Ct})$.

Assessment of surface receptor expression by flow cytometry

Surface expression of Siglec-8 on eosinophils ($CD45^+CD16^-$ granulocytes) was quantified *ex vivo* by multiparameter flow cytometry using fresh whole blood as previously described[12]. Further details of the antibodies and gating strategy are provided in the online supplement.

Measurement of soluble receptor levels in serum

Serum levels of sSiglec-8 were quantified in undiluted, coded (blinded) serum samples using a sandwich ELISA employing paired capture (clone 1H10, mouse IgG1 to domain 3) and

detection (biotinylated clone 2C4, mouse IgG1 to domain 1) antibodies, streptavidin-HRP and Super AquaBlue. All assays were performed in triplicate. Values were calculated based on a standard curve using Siglec-8-Ig. The range of detection of sSiglec-8 in serum was 0.5–60.0 ng/mL.

Histopathologic scoring of esophageal biopsies

Archived unstained slides were obtained from the mid and distal esophagus and blinded prior to immunohistochemistry for tryptase (Cat#MS-1216-P, FisherScientific, Hampton, NH, USA). Peak mast cells per 40x high powered field (MC/hpf) were quantified in all epithelial regions, including the basal zone. Papillae were excluded, along with the lamina propria. Degranulated mast cells were counted as cells with dispersed granules outside the cell as compared to intact cells with compact shape and dense granules (Supplementary Figure 1).

Eosinophil cell death assay

Purified eosinophils were suspended at 2×10^6/mL in culture medium in 96-well U bottom plates and incubated overnight at 37°C, 5% CO_2 with or without IL-5 (30 ng/mL) prior to a second overnight incubation with anti-Siglec-8 antibodies or their respective isotype controls. The addition of IL-5 to these cultures enhances the ability of Siglec-8 crosslinking by anti-Siglec-8 mAb as previously reported[3–5]. The cells were subsequently washed, stained with Annexin-V-FITC/7-AAD (BD Biosciences) and acquired by flow cytometry (LSRII, BD).

In vitro NK-cell mediated eosinophil killing assay

The NK killing assay was performed as previously described[12]. Purified eosinophils (8×10^4) were incubated with autologous NK cells in culture medium at an effector/target ratio of 5:1 in 96-well U-bottom plates at 37°C in the presence or absence of 10 μg/mL c2E2 IgG1 or c2E2 IgG4 or their respective isotype controls. After 4 hours, cells were stained with Annexin-V and 7-AAD. Samples were acquired and analyzed on a flow cytometer (LSRII, BD Biosciences). Eosinophils were identified based on their granularity (high side scatter). ADCC was determined by gating on Annexin-V^+ target cells.

Peripheral blood eosinophil depletion assay

After erythrocyte lysis using ACK lysing buffer, PBL were incubated in culture medium at a final concentration of 10×10^6/ml in 96-well U bottom plates (1 million/well). Anti-Siglec-8 antibodies or their respective isotype controls were added for 4 hours at 37°C, 5% CO_2. The cells were washed once before staining in PBS-1% BSA with anti-CD16 and anti-VLA4 antibodies (BD Biosciences) and 7-AAD at 4°C for 20 minutes. Samples were acquired on the LSRII flow cytometer (20 seconds per tube). Live eosinophils were gated and counted as $CD16^{neg}$ $VLA4^+$7-AAD^{neg} granulocytes.

Generation and treatment of humanized mice

$CD34^+$ cells were enriched from umbilical cord blood of deidentified donors by the Translational Core Laboratory at Cincinnati Children's Hospital. Humanized mice were

generated by engrafting 10-week-old NSGS mice (non-obese diabetic/severe combined immunodeficiency, transgenic for human stem cell factor [SCF], IL-3, and granulocyte macrophage colony stimulating factor [GM-CSF]) obtained from Jackson Laboratory with human CD34-selected umbilical cord cells (50,000 cells per mouse intravenously) following pretreatment with busulfan (30 mg/kg)[13]. Reconstitution of Siglec-8-expressing human eosinophils was confirmed by flow cytometry (at week 9 post-engraftment, the mice had 3.04% ± 0.83% Siglec-8$^+$ cells among human CD45$^+$ cells (Supplementary Figure 2A and B). These cells also expressed human eosinophil peroxidase (EPX) by flow cytometry and morphologically resembled human eosinophils (Supplementary Methods and Figure 2B). Mice were then treated with 6 doses of human IL-5 (R&D systems, 0.2 μg/mouse intraperitoneally over two days, Supplementary Figure 2C), followed by a single dose of anti-Siglec-8 or isotype-control antibody (0.1 mg/mouse intraperitoneally) at the time of last dose of IL-5. Mice were sacrificed and blood samples collected 18 hours after antibody treatment. This work was approved by the Cincinnati Children's Hospital IACUC, protocols 0D11085 and 3D09061.

Statistical analysis

Statistical analyses were performed using the nonparametric Mann-Whitney U test for comparisons of group means and Fisher's exact test for comparison of proportions. A one-way ANOVA test with post-hoc Bonferroni testing was used for multiple group comparison of soluble Siglec-8 levels. Paired samples were compared using Wilcoxon signed-rank test and correlations were determined using Spearman rank. A *P* value of less than 0.05 was considered statistically significant for all analyses.

Results

Siglec-8 is consistently expressed at similar levels on eosinophils from normal and eosinophilic donors

Siglec-8 was highly expressed on blood eosinophils from all 125 subjects tested (38 ND and 87 EO; GM mfi 383, range 141–595). Despite significantly increased AEC in EO with active disease (Figure 1A), Siglec-8 expression was comparable between ND and EO and was unaffected by treatment status (Figure 1B). Siglec-8 expression was also unchanged on eosinophils from six EO subjects evaluated before and after successful treatment with prednisone (n=5) or imatinib (n=1), despite a dramatic decrease in AEC (Figure 1C). Siglec-8 expression was not correlated with AEC either in the cohort as a whole or among the EO (r=0.02, P=0.81; Figure 1D) and did not vary between clinical subtypes of HES (Supplementary Figure 3).

Siglec-8 transcriptional activity in blood eosinophils is similar between normal and eosinophilic donors

Consistent with surface expression levels, *SIGLEC-8* mRNA expression was comparable in blood eosinophils from ND and EO and was unaffected by treatment status or AEC (Figure 2A, 2B). Based on data from 19 subjects with PCR and flow cytometry data available from the same date, eosinophil mRNA and surface expression of Siglec-8 were correlated (r=0.6, P<0.01; Figure 2C).

Serum soluble Siglec-8 levels in HES do not correlate with eosinophilia

Serum sSiglec-8 levels were detectable (>0.5 ng/mL) in serum from 6/10 ND and 103/129 EO (p=NS, Fisher's exact), and GM levels were comparable between the two groups (1.27 and 1.52 ng/mL, respectively; Figure 3A, B). Three EO subjects had levels above the measurable range of the assay (60 ng/mL). Serum sSiglec-8 levels in 67 subjects on no therapy (9 ND and 58 EO) did not correlate with AEC (Supplementary Figure 4A), and levels were not different among the various clinical subtypes of HES (Supplementary Figure 4B). However, serum sSiglec-8 levels measured before and after treatment in 9 *FIP1L1-PDGFRA*$^{+}$ and 6 *FIP1L1-PDGFRA*^{neg} HES subjects decreased significantly (from GM 2.08 to 0.87; $P<0.01$ and 1.64 to 0.50 ng/mL; $P< 0.05$, respectively; Figures 3C and D), as did AEC. In contrast, serum sSiglec-8 levels from 8 untreated EO and 2 ND measured at two separate time points were comparable (GM 2.17 and 2.10 ng/mL, Figures 3C and D). Serum sSiglec-8 levels were not correlated with eosinophil surface (n=35; Figure 3E) or mRNA (n=27; Figure 3F) expression of Siglec-8.

The possibility that MCs, rather than eosinophils, were the primary source of sSiglec-8 was investigated by examining sSiglec-8 levels in the serum of subjects with SM (Supplementary Table 1). sSiglec-8 levels were <1 ng/mL in serum from all 22 D816V *KIT*-positive SM subjects tested, including 4 D816V *KIT*-positive SM-eo patients with AEC>1900/μL (Figure 3B). To determine if something in the serum of SM donors was interfering with the ELISA, SM serum was added to Siglec-8-Ig (at 1, 3 or 10 ng/mL). This did not alter Siglec-8 levels detected in the ELISA (data not shown).

Soluble Siglec 8 levels in EoE

sSiglec-8 levels were comparable in serum from patients with active EoE (n=21), inactive EoE (n=13), GERD (n=4) and ND (n=7) (Supplementary Table 1; Figure 4A). Serum sSiglec-8 levels were also similar during inactive and active disease in 15 EoE subjects with paired samples available for analysis (Figure 4B). Although serum sSiglec-8 levels were not correlated with peak eosinophil density on esophageal biopsies (Figure 4C) or AEC (Figure 4D) in subjects with EoE, a trend towards a correlation between serum sSiglec-8 levels and peak mast cell density in esophageal biopsies was observed (Figure 4E, 4F).

Anti-Siglec-8 antibodies induce eosinophil cell death in the presence of IL-5

c2E2 IgG1 and c2E2 IgG4 were equally effective in inducing cell death of IL-5-primed purified eosinophils from 8 ND and 28 EO donors after 24 hours. GM %Annexin-V+ eosinophils increased significantly from 12.1 to 60.1% in EO and from 10 to 52.2% in ND in response to c2E2 IgG1 and from 12.5 to 58.1% in EO and from 10 to 52.5% in ND in response to c2E2 IgG4 as compared to matched isotype controls (Figure 5A).

The two antibodies showed similar efficiency in inducing Annexin-V expression on purified eosinophils from the same donor (Supplementary Figure 5) and acted in a dose-dependent manner (Figure 5B). The percentage of 7-AAD^{+} (late apoptotic/necrotic) eosinophils also increased significantly at all antibody doses tested, but remained low (16 ± 4%, n=21, Figure 5C), consistent with the previously described pro-apoptotic effect of anti-Siglec-8 antibodies.

The afucosylated IgG1 anti-Siglec-8 antibody, c2E2 IgG1*, enhances eosinophil killing through ADCC*

NK cell–mediated killing of eosinophils in the absence of exogenous IL-5 was assessed *in vitro* after 4 hours using purified eosinophils and NK cells from 8 ND and 15 EO (Figure 6A). While the GM % of Annexin-V$^+$ eosinophils increased in the presence of c2E2 IgG1 (from 21.1% to 38.2% for EO, $P<0.001$ and from 22.8% to 53.7% for ND, $P<0.01$), c2E2 IgG4 had no effect (Figure 6B). Although Annexin-V positivity of purified eosinophils in the presence of NK cells at 4 hours was variable in the absence of anti-Siglec-8 antibodies, neither c2E2 IgG1 nor c2E2 IgG4 had a significant effect on the viability of eosinophils or NK cells when incubated separately under the same conditions. (Supplementary Figure 6).

c2E2 IgG4 *depletes eosinophils from PBL preparations in the absence of exogenous IL-5 only in eosinophilic donors*

Given the potential effects of eosinophil priming by mediators produced by bystander cells, especially in eosinophilic subjects, the ability of c2E2 IgG4 to deplete eosinophils from ND and EO was assessed using PBL preparations in the presence and absence of IL-5. As in prior experiments with purified eosinophils, c2E2 IgG4 induced significant eosinophil depletion after 4 hr in the presence of IL-5 (30 ng/mL) at antibody concentrations ranging from 0.01 μg/mL to 10 μg/mL (Figure 7A, B). Although mean eosinophil depletion by c2E2 IgG4 was not significant in the absence of IL-5 in ND (Figure 7A), all 5 EO donors demonstrated some degree of depletion in response to c2E2 IgG4 with mean % eosinophils remaining 63.1±10.4 compared to 90.8±4.5 for isotype control at 10 μg/mL ($p<0.05$; Figure 7B).

Eosinophil depletion by anti-Siglec-8 antibodies in humanized mice in vivo

To assess the effect of anti-Siglec-8 antibodies *in vivo*, humanized NSGS mice were treated with rhIL-5 to increase the number of circulating human eosinophils prior to antibody administration (Figure 7C). Mean % of EPX$^+$ eosinophils decreased from 8.4 to 2.7 and 3.6 with m2E2 IgG1 (murine equivalent of human IgG1) and c2E2 IgG1, respectively ($p<0.05$; Figure 7D).

Discussion

Although the recent approvals of monoclonal antibodies to IL-5 (mepolizumab and reslizumab) and IL-5 receptor α (benralizumab) for treatment of severe asthma have begun to address the unmet need for safe and effective therapies for eosinophil-associated disorders (EAD), additional agents are clearly needed. In the present study, we examined the expression and modulation of Siglec-8 on eosinophils from a large cohort of normal and eosinophilic donors and tested the activity of two different anti-Siglec-8 antibodies in depleting eosinophils *in vitro* and *in vivo* in a humanized mouse model.

The efficacy of surface receptor antibodies depends on sufficient and reliable receptor expression on target cells. Downmodulation of IL-5 receptor α expression on eosinophils by factors associated with eosinophilia, eosinophil recruitment and eosinophil activation has been described[14–16] and could potentially limit activity of currently approved anti-eosinophil

biologics in some settings. In contrast, consistent with prior reports in a small number of subjects[10], Siglec-8 was consistently expressed on eosinophils from all donors tested, and surface levels did not correlate with AEC or differ by clinical subtype or treatment status (Figure 1, Supplementary Figure 3).

Similar to other cell surface receptors, Siglec-8 has a soluble form that is measurable in serum, where it could bind anti-Siglec-8 antibodies and potentially impair treatment efficacy. Although sSiglec-8 was detectable in serum from most eosinophilic subjects tested, levels were low and would be unlikely to interfere with the efficacy of anti-Siglec-8 antibodies. Equally important, sSiglec-8 levels were not correlated with AEC or HES subtype, suggesting that sSiglec-8 levels would not impair the efficacy of anti-Siglec-8 antibody therapy in patients with the most severe disease.

The lack of correlation between sSiglec-8 levels and AEC suggests that peripheral blood eosinophils may not be the only source of sSiglec-8 in eosinophilic patients. Although both AEC and sSiglec-8 levels decreased dramatically in patients with HES post-treatment (Figure 3F), successful treatment likely has effects on other cells, including tissue eosinophils and mast cells expressing Siglec-8. The lack of correlation between sSiglec-8 levels and tissue eosinophils in EoE and trend towards a correlation with tissue mast cells supports a possible role for mast cells as a source of sSiglec-8 in EoE. Surprisingly, sSiglec-8 levels were not measurable in patients with D816V *KIT*-positive SM (with or without marked eosinophilia) despite increased numbers of mast cells and documentation of surface expression of Siglec-8 on mast cells and eosinophils in this setting[10]. Whether the D816V *KIT* mutation, recently reported to impact the redox status of mutation-bearing cells[17], prevents receptor shedding remains to be elucidated.

The consistent level of surface expression of Siglec-8 on eosinophils and lack of evidence for downmodulation or shedding of receptor in the setting of eosinophil activation support targeting of Siglec-8 as a treatment for EADs. To further explore the utility of this strategy, the ability of two chimeric anti-Siglec-8 antibodies, c2E2 IgG4 and c2E2 IgG1, to induce eosinophil cell death *in vitro* was explored. Both antibodies have identical variable regions and would be expected to induce eosinophil apoptosis in the presence of IL-5. Although c2E2 IgG1 has the theoretical advantage of mediating enhanced NK-mediated ADCC independent of IL-5 in humans, NK cell function is not reconstituted in NSG mice[18].

As expected, c2E2 IgG1 and c2E2 IgG4 were equally effective in inducing eosinophil apoptosis *in vitro* in the presence of IL-5. Moreover, eosinophil apoptosis was comparable between ND and EO, irrespective of treatment status. Depletion of IL-5-elicited human eosinophils was also observed with both m2E2 IgG1 (the murine equivalent of c2E2 IgG4) and c2E2 IgG1. Although eosinophil depletion was similar in subjects with different clinical variants of HES, eosinophils from the single subject with *FIP1L1-PDGFRA* myeloid neoplasm included in the analysis were resistant to antibody treatment prior to (but not after) imatinib therapy (Supplementary Figure 7).

Similar to afucosylated IgG1 antibodies targeting other eosinophil surface receptors, including EMR1[12] and IL-5Rα[8], c2E2 IgG1 (but not c2E2 IgG4) induced killing of ND

eosinophils by NK cells *in vitro* in the absence of exogenous IL-5. In contrast, c2E2 IgG4 depleted eosinophils from EO (and some ND) in the PBL assay in the absence of exogenous IL-5. This difference in c2E2 IgG4 activity could be due to *in vivo* priming of eosinophils from EO donors and/or secretion of IL-5, GM-CSF or other eosinophil-priming cytokines by other cells, including Th2 cells and ILC2s, present in the PBL cultures. Although the clinical implications of these *in vitro* findings remain to be proven, the data suggest that patients with IL-5-driven eosinophilia, such as those with lymphocyte variant HES, may be most likely to benefit from anti-Siglec-8 IgG4 antibody therapy. Conversely, these antibodies may be less effective in the treatment of myeloid forms of HES, including *PDGFRA*-associated myeloid neoplasms, where the eosinophilia is less dependent on IL-5 and eosinophils may be relatively resistant to ADCC (Supplemental Figure 5). Although mepolizumab and reslizumab target IL-5 directly and might be expected to be effective in the same subset of patients as anti-Siglec antibodies, data from several studies of mepolizumab suggest that patients with the highest IL-5 levels (those with lymphocytic variant HES) are actually less responsive to mepolizumab than those with idiopathic disease[19], perhaps due to saturation of the antibody.

In summary, data from a large cohort of normal and eosinophilic subjects confirm that Siglec-8 is highly expressed on the surface of blood eosinophils irrespective of AEC, underlying etiology of the eosinophilia or treatment status. Serum sSiglec-8 levels did not correlate with AEC or tissue eosinophilia and should not jeopardize the efficacy of anti-Siglec-8 antibodies in eosinophilic patients. Finally, c2E2 IgG1 and c2E2 IgG4 induced apoptosis of eosinophils from normal and eosinophilic subjects *in vitro* and eosinophils of IL-5-treated humanized mice *in vivo*. Taken together, these data support further development of anti-Siglec-8 antibodies for the treatment of EAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank the clinical staff of the Laboratory of Parasitic Diseases for assistance in procuring subject data and samples at the NIH and Amir Kagalwalla, MD, Barry Wershil and Steve Ackerman, PhD for their assistance in obtaining patient specimens at Ann & Robert H. Lurie Children's Hospital of Chicago. The authors would additionally like to thank Mark Wunderlich and Dr. James Mulloy for assistance with the generation of humanized mice.

Funding

This study was supported in part by the Division of Intramural Research, NIAID, NIH. Development of the soluble Siglec-8 ELISA in the Bochner lab was supported in part by NIH grant AI097073. Collection of patient specimens/data at Ann & Robert H. Lurie Children's Hospital funded by American Partnership for Eosinophilic Diseases (APFED) and Buckeye Foundation. Dr. Wechsler is funded by K08 1K08DK097721–01. Anti-Siglec-8 antibodies and funding for NZ for this project were provided by Allakos Inc.

Abbreviations

7-AAD	7-aminoactinomycin D
AEC	Absolute eosinophil count

A.U.	Arbitrary units
ADCC	Antibody-dependent cellular cytotoxicity
c2E2 IgG4	Chimeric 2E2 IgG4
c2E2 IgG1	Chimeric 2E2 IgG1
CM	Culture Medium
EAD	Eosinophil-associated diseases
EO	Eosinophilic donors
EoE	Eosinophilic esophagitis
GM	Geometric mean
HES	Hypereosinophilic syndromes
MC	Mast cell
Mfi	Mean fluorescence intensity
ND	Normal donors
PBL	Peripheral blood leukocytes
ROS	Reactive Oxygen Species
rh	Recombinant human
sSiglec-8	Soluble Siglec-8
SM	Systemic mastocytosis

Bibliography

1. Legrand F, Klion AD. Biologic therapies targeting eosinophils: current status and future prospects. J Allergy Clin Immunol Pract 2015;3:167–74. [PubMed: 25754717]
2. Klion AD. How I treat hypereosinophilic syndromes. Blood 2015;126:1069–77. [PubMed: 25964669]
3. Nutku E, Aizawa H, Hudson SA, Bochner BS. Ligation of Siglec-8: a selective mechanism for induction of human eosinophil apoptosis. Blood 2003;101:5014–20. [PubMed: 12609831]
4. Kano G, Almanan M, Bochner BS, Zimmermann N. Mechanism of Siglec-8-mediated cell death in IL-5-activated eosinophils: role for reactive oxygen species-enhanced MEK/ERK activation. J Allergy Clin Immunol 2013;132:437–45. [PubMed: 23684072]
5. Carroll DJ, O'Sullivan JA, Nix DB, Cao Y, Tiemeyer M, Bochner BS. Sialic acid-binding immunoglobulin-like lectin 8 (Siglec-8) is an activating receptor mediating β2-integrin-dependent function in human eosinophils. J Allergy Clin Immunol 2018;141:2196–2207 [PubMed: 28888781]
6. Na HJ, Hudson SA, Bochner BS. IL-33 enhances Siglec-8 mediated apoptosis of human eosinophils. Cytokine 2012;57:169–74. [PubMed: 22079334]
7. Yokoi H, Choi OH, Hubbard W, Lee H-S, Canning BJ, Lee HH, et al. Inhibition of FcepsilonRI-dependent mediator release and calcium flux from human mast cells by sialic acid-binding immunoglobulin-like lectin 8 engagement. J Allergy Clin Immunol 2008;121:499–505.e1. [PubMed: 18036650]

8. Kolbeck R, Kozhich A, Koike M, Peng L, Andersson CK, Damschroder MM, et al. MEDI-563, a humanized anti-IL-5 receptor alpha mAb with enhanced antibody-dependent cell-mediated cytotoxicity function. J Allergy Clin Immunol 2010;125:1344–1353.e2. [PubMed: 20513525]
9. Wu Y, Li JJ, Kim HJ, Liu X, Liu W, Akhgar A, et al. A neutralizing antibody assay based on a reporter of antibody-dependent cell-mediated cytotoxicity. AAPS J 2015;17:1417–26. [PubMed: 26205082]
10. Hudson SA, Herrmann H, Du J, Cox P, Haddad E-B, Butler B, et al. Developmental, malignancy-related, and cross-species analysis of eosinophil, mast cell, and basophil siglec-8 expression. J Clin Immunol 2011;31:1045–53. [PubMed: 21938510]
11. Na HJ, Hamilton RG, Klion AD, Bochner BS. Biomarkers of eosinophil involvement in allergic and eosinophilic diseases: review of phenotypic and serum markers including a novel assay to quantify levels of soluble Siglec-8. J Immunol Methods 2012;383:39–46. [PubMed: 22683541]
12. Legrand F, Tomasevic N, Simakova O, Lee C-CR, Wang Z, Raffeld M, et al. The eosinophil surface receptor epidermal growth factor-like module containing mucin-like hormone receptor 1 (EMR1): a novel therapeutic target for eosinophilic disorders. J Allergy Clin Immunol 2014;133:1439–47, 1447.e1. [PubMed: 24530099]
13. Wunderlich M, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M, et al. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. Leukemia 2010;24:1785–8. [PubMed: 20686503]
14. Wilson TM, Maric I, Shukla J, Brown M, Santos C, Simakova O, et al. IL-5 receptor α levels in patients with marked eosinophilia or mastocytosis. J Allergy Clin Immunol 2011;128:1086–92.e1. [PubMed: 21762978]
15. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H, et al. Decreased expression of membrane IL-5 receptor alpha on human eosinophils: II. IL-5 down-modulates its receptor via a proteinase-mediated process. J Immunol 2002;169:6459–66. [PubMed: 12444155]
16. Liu LY, Sedgwick JB, Bates ME, Vrtis RF, Gern JE, Kita H, et al. Decreased expression of membrane IL-5 receptor alpha on human eosinophils: I. Loss of membrane IL-5 receptor alpha on airway eosinophils and increased soluble IL-5 receptor alpha in the airway after allergen challenge. J Immunol 2002;169:6452–8. [PubMed: 12444154]
17. Kim D-K, Beaven MA, Kulinski JM, Desai A, Bandara G, Bai Y, et al. Regulation of Reactive Oxygen Species and the Antioxidant Protein DJ −1 in Mastocytosis. PLoS ONE 2016;11:e0162831. [PubMed: 27611333]
18. Herndler-Brandstetter D, Shan L, Yao Y, Stecher C, Plajer V, Lietzenmayer M, et al. Humanized mouse model supports development, function, and tissue residency of human natural killer cells. Proc Natl Acad Sci U S A 2017;114:E9626–34. [PubMed: 29078283]
19. Roufosse F, de Lavareille A, Schandené L, Cogan E, Georgelas A, Wagner L, et al. Mepolizumab as a corticosteroid-sparing agent in lymphocytic variant hypereosinophilic syndrome. J Allergy Clin Immunol 2010;126:828–835.e3. [PubMed: 20810155]

Key Messages

- Siglec-8 is consistently expressed on blood eosinophils from normal and eosinophilic subjects, and is not correlated with absolute eosinophil count, disease activity or serum levels of soluble Siglec-8.
- Anti-Siglec-8 antibodies induce cell death of eosinophils from normal and eosinophilic donors *in vitro* and in a humanized mouse model of IL-5-induced eosinophilia.

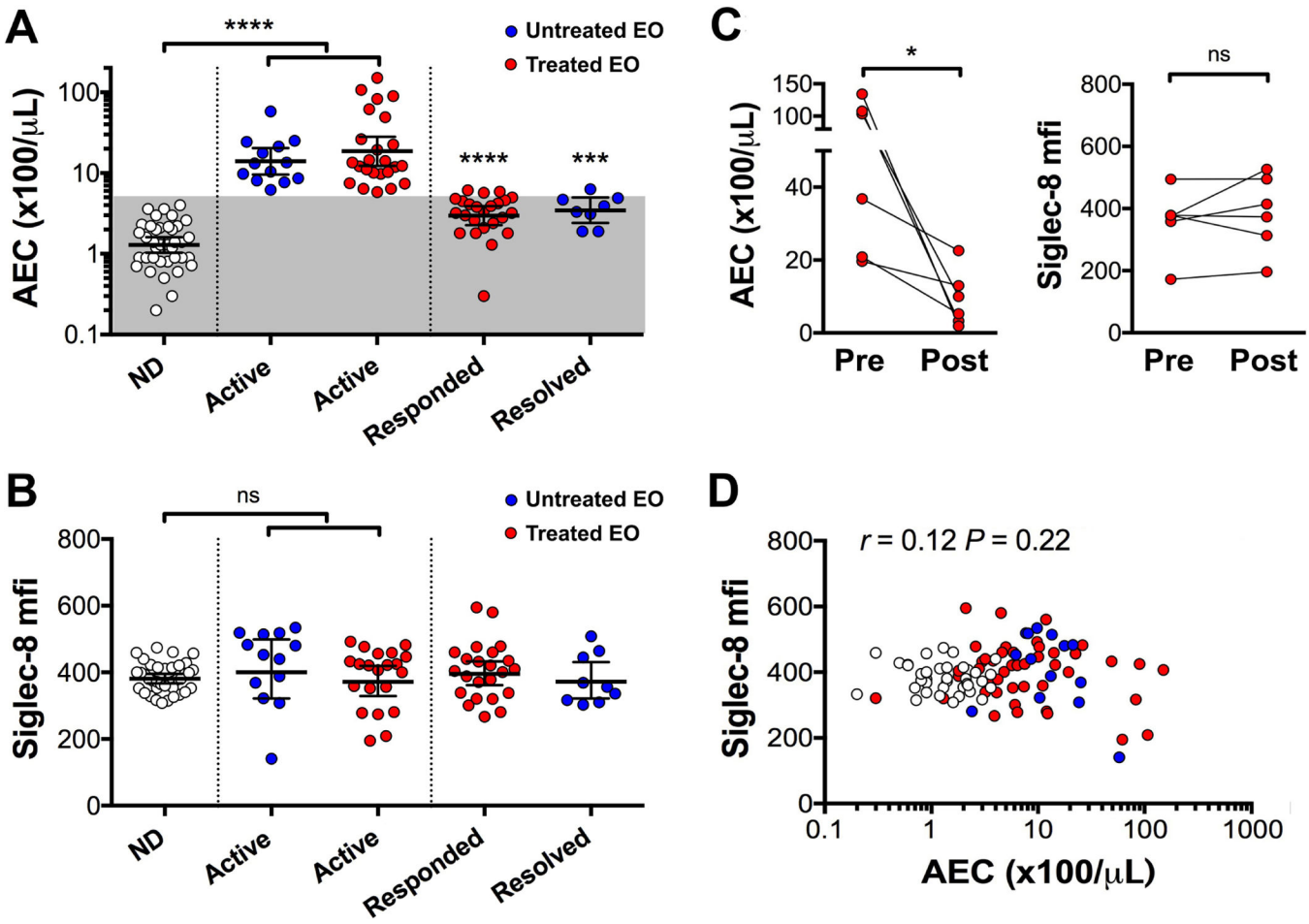


FIG 1.

Eosinophil count and surface expression of Siglec-8. *A*, Absolute eosinophil count (AEC) and *B*, eosinophil surface expression of Siglec-8 expressed as mean fluorescence intensity (mfi) of normal (ND; white circles) and eosinophilic subjects (EO) on treatment (blue circles) or untreated (red circles). EO subjects are classified as Active (AEC≥1500/μL or marked tissue eosinophilia and AEC>500/μL), Responded (clinically improved with AEC≤1000/μL on therapy) or Resolved (clinically improved with AEC≤1000/μL on no therapy). The horizontal bars represent geometric means with 95% CI. Gray shading indicates AEC<500/μL. *C*, Changes in AEC and Siglec-8 mfi pre-and post-treatment in EO subjects. **P*<0.05, Wilcoxon matched pairs, signed rank test, *D*, Correlation between AEC and Siglec-8 mfi on eosinophils from ND (white circles) and EO subjects on treatment (blue circles) or untreated (red circles). Spearman correlation *P*=NS.

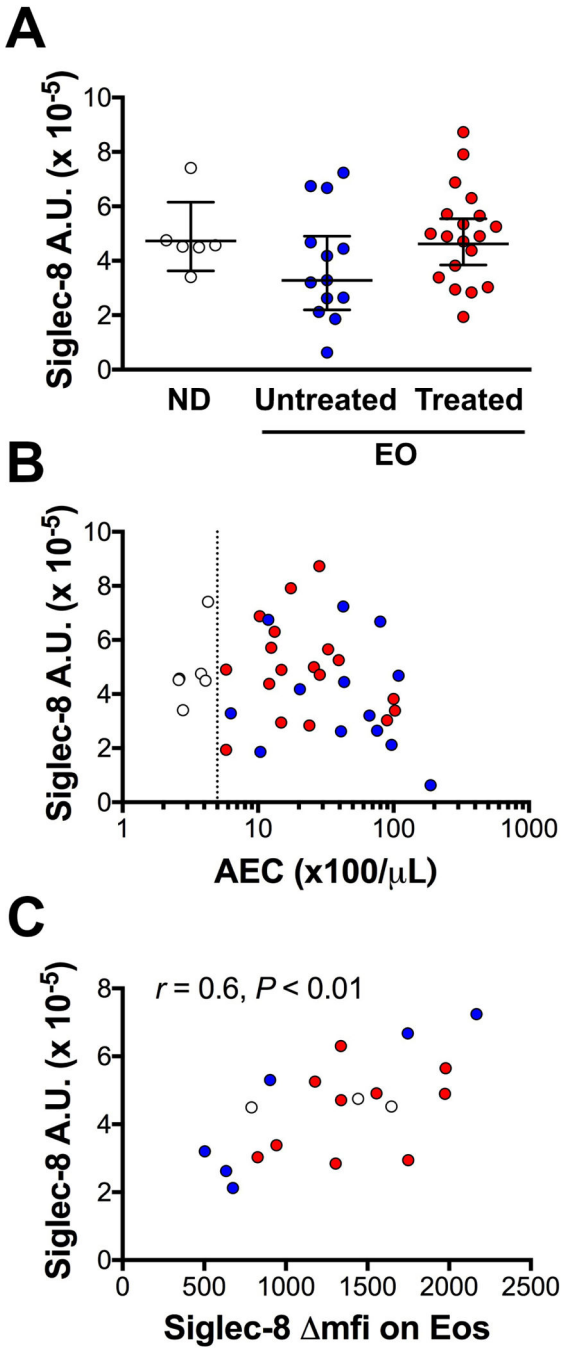


FIG 2.

SIGLEC-8 mRNA expression in blood eosinophils. *A*, Eosinophil levels of Siglec-8 mRNA in ND (white circles), and EO, untreated (blue circles) and on treatment (red circles). Geometric means with 95% CI are indicated by horizontal lines. *B*, Correlation between AEC and *SIGLEC-8* mRNA. The vertical dotted line indicates AEC=500/μL. *C*, Correlation between mRNA and eosinophil surface expression of Siglec-8 (Δmfi); $P<0.01$, Spearman correlation.

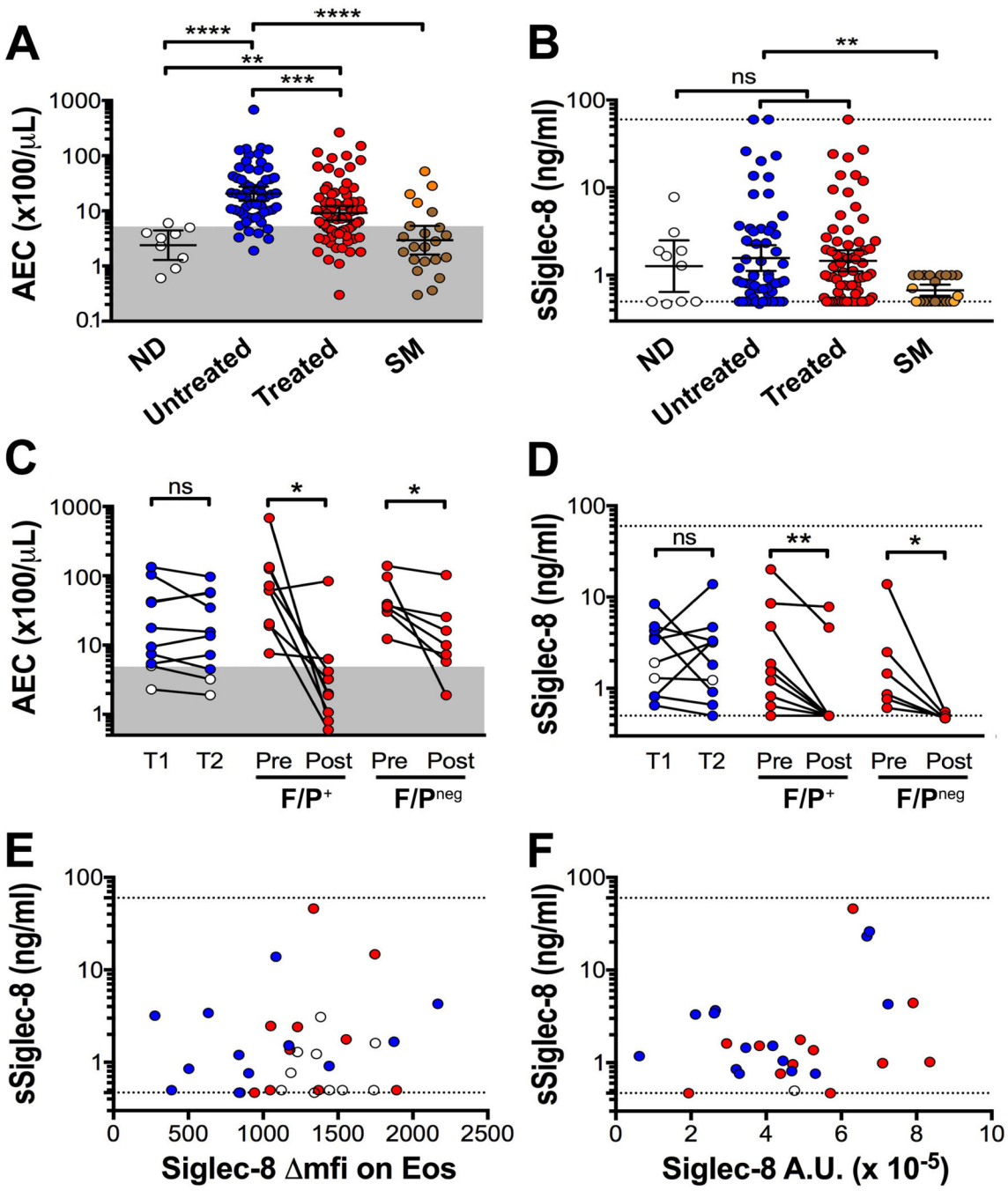


FIG 3.

Serum soluble Siglec-8 levels from various donors. *A,* AEC and *B*, soluble serum levels of Siglec-8 in ND (white circles), untreated EO (blue circles), EO on treatment (red circles), systemic mastocytosis (SM) patients (brown circles) and SM-Eo (orange circles). Geometric means with 95% CI are indicated by horizontal lines. Changes in AEC (*C)*, and changes in serum sSiglec-8 (*D*) at two time points (T1 and T2) in ND (white circles) and EO subjects (blue circles) without a change in treatment and pre-and post-treatment in F/P^{+} and F/P^{neg} EO subjects treated for 1–3 months with imatinib 400 mg daily (F/P^{+}; n=7), prednisone 5–

35 mg daily (F/P^{neg}; n=5) or hydroxyurea 500 mg daily (F/P^{neg}; n=1) (red circles) (Wilcoxon matched pairs, signed rank test, ** $P<0.01$ and * $P<0.05$). *E*, Correlation between soluble Siglec-8 levels and surface expression of Siglec-8. *F*, Correlation between soluble Siglec-8 levels *SIGLEC-8* mRNA levels. Gray shading in panels A and E indicate AEC<500/μL. Dotted horizontal lines in panels B-D and F-H represent the upper and lower limits of detection for soluble Siglec-8.

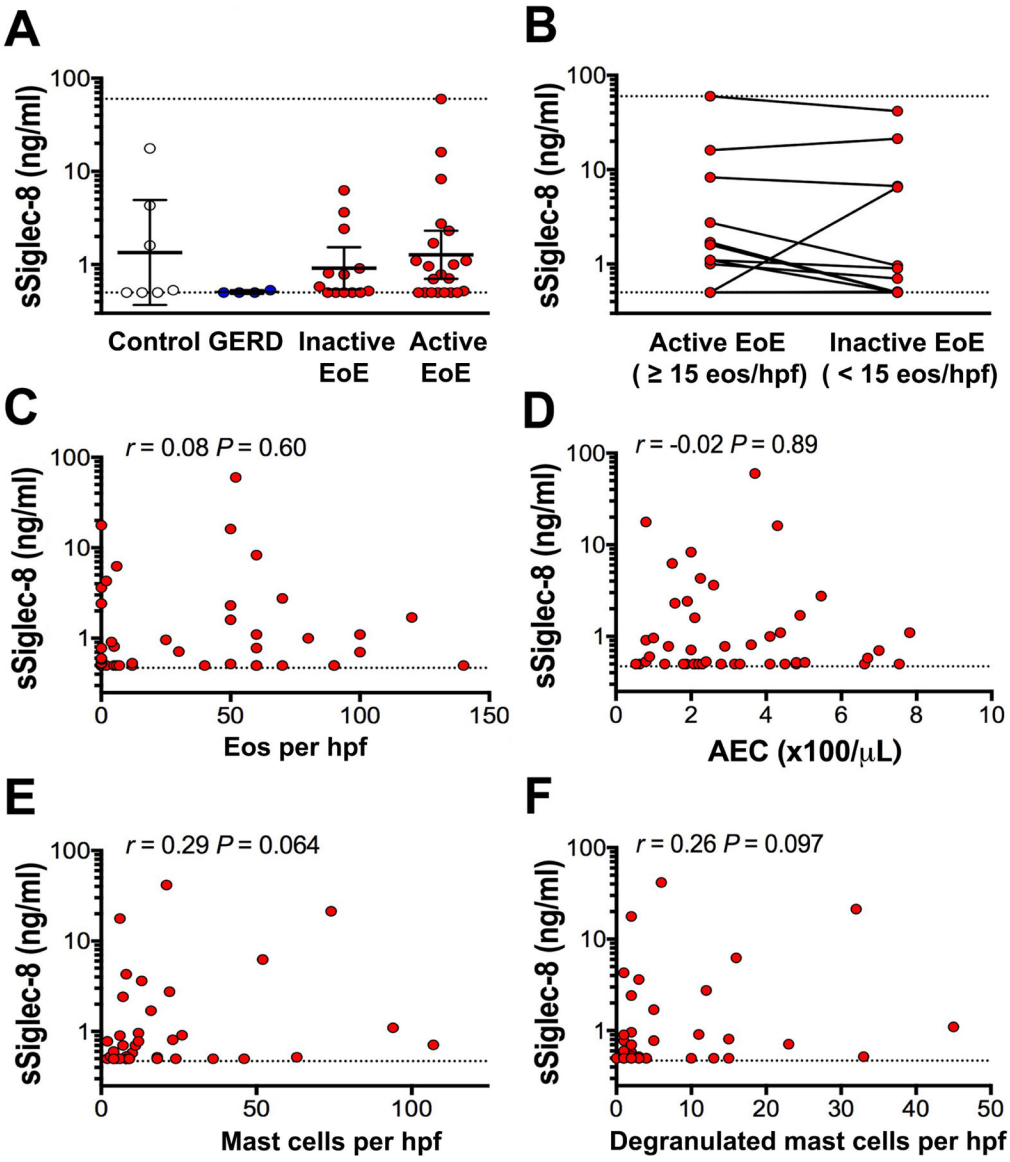


FIG 4.

Soluble Siglec-8 levels in subjects with eosinophilic esophagitis (EoE). *A,* Serum levels of sSiglec8 in Control (white circles), GERD (blue circles), Inactive and Active EoE (red circles). *P*=ns, one-way ANOVA with post hoc Bonferroni, *B,* Serum sSiglec8 levels in paired samples from EoE subjects during active and inactive disease. *P*=ns, Wilcoxon matched pairs, signed rank test. *C-F,* Correlation between serum Siglec-8 levels and peak intra-epithelial eosinophils per high powered field (eos/hpf) in esophageal biopsies (*C*), AEC (*D*), intraepithelial mast cells/hpf (*E*) and degranulated mast cells/hpf (*F*) in esophageal biopsies. Significance was assessed by Spearman correlation. Dotted horizontal lines in panels A and B represent the upper and lower limits of detection for soluble Siglec-8.

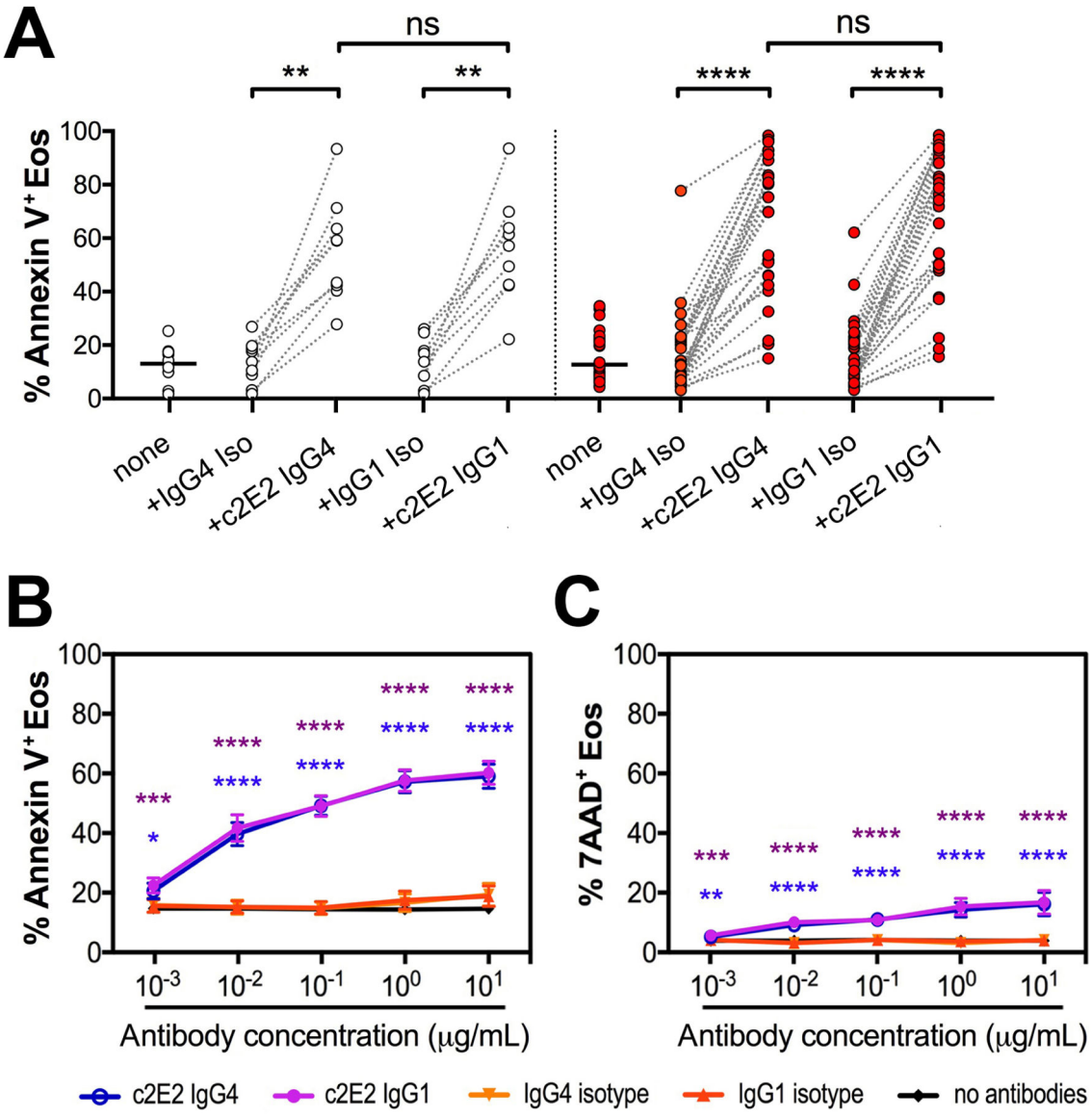


FIG 5.

Eosinophil apoptosis induced by c2E2 IgG1 and c2E2 IgG4 antibodies to Siglec-8. *A,* Annexin-V+ eosinophils from ND (white circles; n=8) and EO (red circles; n=29) after overnight incubation in the presence of c2E2 IgG1 or c2E2 IgG4 (10 μg/mL) following pre-incubation with IL-5 (30 ng/mL for 18h). *B* and *C*, Dose-dependent effect of anti-Siglec-8 antibodies (10^{-3} to 10 μg/mL) on eosinophil cell death in presence of IL-5. Mean±SEM % Annexin-V^+ or % 7-AAD^+ (n=36) in the presence of c2E2 IgG4 or c2E2 IgG1 were compared to their respective isotype controls. Wilcoxon matched pairs, signed rank test (* P<0.05, ** P<0.01, *** P<0.001; ****, P<0.0001).

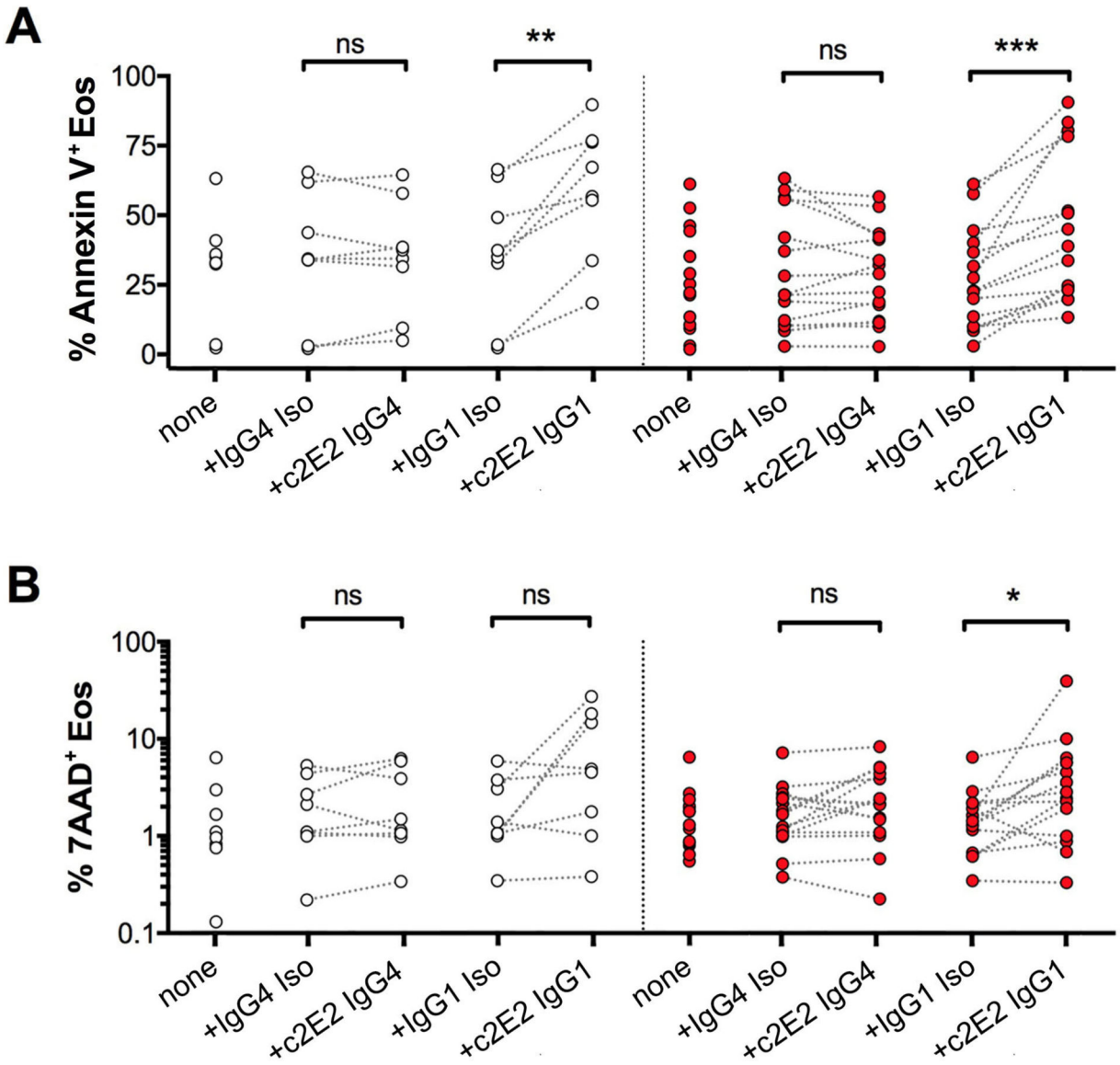


FIG 6.

Eosinophil killing in the presence of NK cells and c2E2 IgG1 and c2E2 IgG4. *A and B*, *In vitro* NK killing assay performed on eosinophils from ND (white circles; n=8) and EO (red circles, n=14) after 4 hrs incubation in presence of anti-Siglec-8 antibodies (10 μg/mL). Annexin-V (*A*) and 7-AAD (*B*) staining is represented for each subject. (*, $P<0.05$, **, $P<0.01$; ***, $P<0.001$, Wilcoxon test).

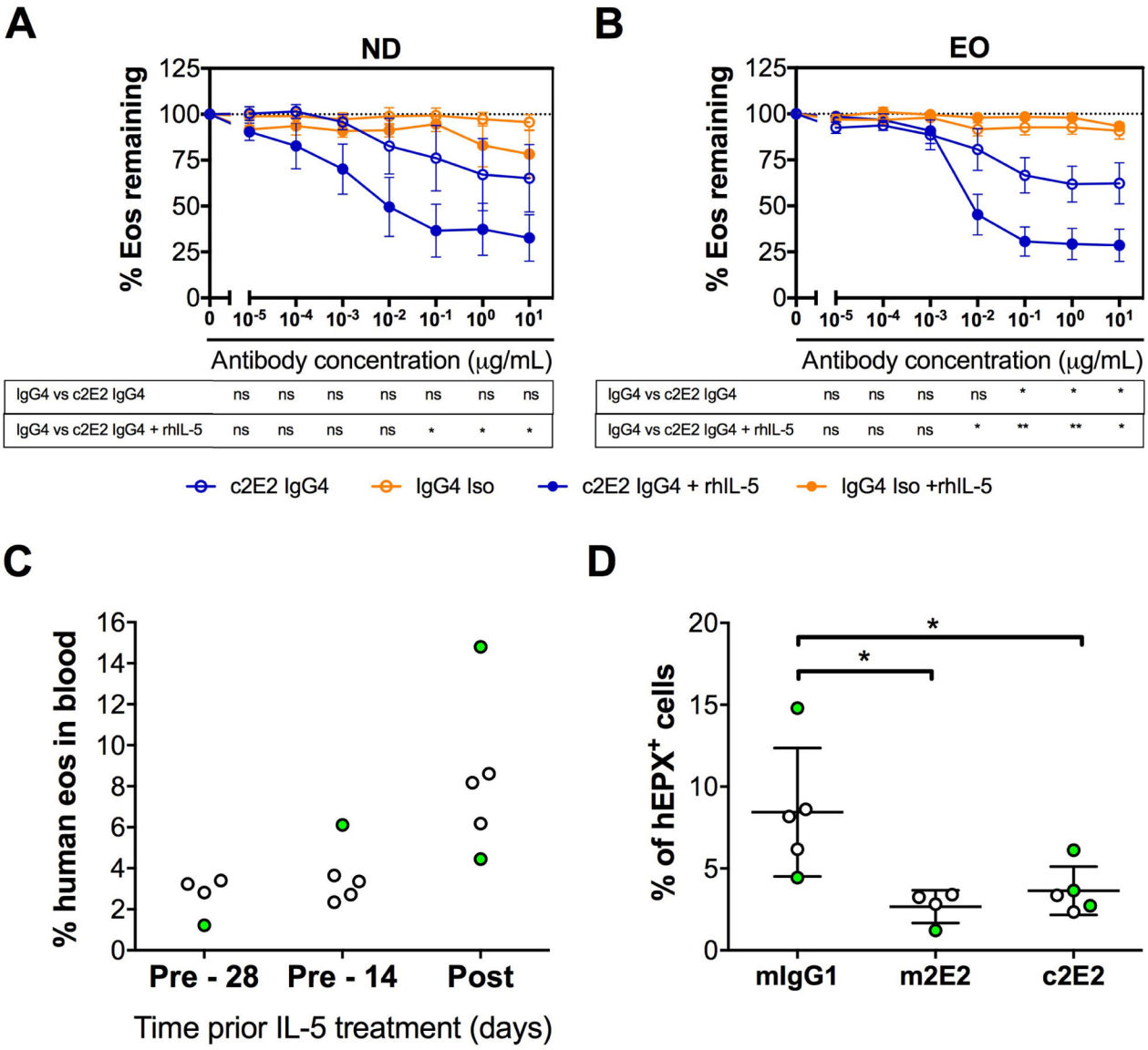


FIG 7.

Eosinophil depletion from peripheral blood leucocyte preparations induced by c2E2 IgG4 *in vitro* and *in vivo*. *A,B*, Dose-dependent effect of c2E2 IgG4 (10^{-5} to 10μg/mL) on eosinophil depletion after overnight incubation in the presence (closed circles) and absence of IL-5 (open circles) in ND (A) and EO (B). Data are expressed as the % of eosinophils remaining normalized to the % of eosinophils in culture medium alone (n=5 per group, graphs show mean+/−SEM). Eosinophil depletion in the presence of c2E2 IgG4 (blue) was compared to the respective isotype control (orange). **P*<0.05, ***P*<0.001. *C,* Percentage of blood eosinophils detected by flow cytometry in engrafted humanized NSGS mice prior to and following administration of hIL-5 (***, *P*<0.001, Mann Whitney test). *D*, Percentage of human ($hEPX^+$) eosinophils 18 hours post-treatment with c2E2 IgG1, m2E2 IgG1 (equivalent of human IgG4) or isotype control antibody (mIgG1). (*, *P*<0.05, Wilcoxon test,

n=5 mice). Engraftment of mice with $CD34^+$ cells from donor 1 are indicated by white circles and from donor 2 by green circles.