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IgE-Binding Monocytes Have an Enhanced Ability to Produce IL-8 (CXCL8) in Animals with Naturally Occurring Allergy

Elisabeth M. Larson, Susanna Babasyan, and Bettina Wagner

IL-8 is a potent chemokine that recruits neutrophils and basophils to promote inflammation in many species. IL-8 is produced by many cell types, including monocytes. In this study, we report a novel role for IgE-binding monocytes, a rare peripheral immune cell type, to promote allergic inflammation through IL-8 production in a horse model of natural IgE-mediated allergy. We developed a mAb with confirmed specificity for both recombinant and native equine IL-8 for flow cytometric analysis. Equine IL-8 was produced by CD14⁺/MHC class II⁺/CD16⁺ monocytes, including a subpopulation of IgE-binding monocytes, following stimulation with LPS. In addition, IgE cross-linking induced IL-8 production by both peripheral blood basophils and IgE-binding monocytes. IL-8 production was compared between healthy horses and those with a naturally occurring IgE-mediated skin allergy, *Culicoides* hypersensitivity. Allergic horses had significantly higher percentages of IL-8⁺ IgE-binding monocytes after IgE cross-linking. In contrast, frequencies of IL-8⁺ basophils after IgE cross-linking were similar in all horses, regardless of allergic disease, highlighting IgE-binding monocytes as a novel source of IL-8 during allergy. We concluded that IgE-binding monocytes from allergic individuals have an increased capacity for IL-8 production and likely contribute to the recruitment of innate immune cells during IgE-mediated allergy and promotion of inflammation during repeated allergen contact. *The Journal of Immunology*, 2021, 206: 2312–2321.

Interleukin 8, also known as CXCL8, is a chemokine involved in the recruitment of inflammatory cells. In humans, IL-8 was originally described as a “neutrophil-attracting peptide” (1, 2) and is well established in its recruitment of neutrophils and also basophils to sites of inflammation (3–7). Human IL-8 is produced by multiple cell types, including monocytes, endothelial cells, epithelial cells, fibroblasts, keratinocytes, synovial cells, and chondrocytes after LPS, TNF, or IL-1 stimulation (8, 9), and binds to human CXCR1 and CXCR2 chemokine receptors (10–12). Human mast cells, basophils (5–7, 13–18), and IgE-binding monocytes (19, 20) also produce IL-8 after IgE cross-linking, suggesting a role for IL-8 in the context of IgE-mediated allergic inflammation.

Rodents do not express any known direct homologues of IL-8 (21, 22). They do, however, express homologues of human CXCR1 and CXCR2 (21, 23, 24). The chemokines CXCL1/KC, CXCL2/MIP-2, CXCL5/LIX, and CXCL6/GCP-2 have been studied as functional homologues in the recruitment of neutrophils in mice because of their binding through the same receptors (21, 22). As a result, mice have limitations for the study of IL-8 in the context of inflammatory diseases. In contrast, horses naturally produce IL-8 and also exhibit many inflammatory diseases that are similar to those in humans (9, 25). We therefore used a horse model for investigating IL-8 production during allergic inflammation.

Equine IL-8 is derived from a 101 aa precursor that can be cleaved into multiple different biologically active variants, the most prevalent being 88 aa long, which has 72.45% homology with human IL-8 (3). In horses, IL-8 mRNA production has

primarily been studied in the context of recurrent airway obstruction (RAO), also known as heaves or severe equine asthma. RAO is characterized by bronchoconstriction and hyperreactivity, pulmonary neutrophilia, and enhanced airway mucus production (26). It is expected that IL-8 molecules produced in the lungs of horses with RAO recruit additional inflammatory mediators, thereby enhancing disease (27–30). A rabbit anti-equine IL-8 mAb (30, 31) was previously developed to detect IL-8 in horses with RAO, but confirmation of IL-8 specificity was not performed. Additional studies showed no differences in IL-8 mRNA between horses with and without RAO (32, 33), emphasizing the importance of measuring IL-8 at the protein level, as mRNA abundance does not always correlate into translated protein. IL-8 production and chemotactic ability in the context of other equine inflammatory diseases has not yet been studied.

In this study, we analyzed the production of equine IL-8 in the context of an allergic disease, *Culicoides* hypersensitivity. This is a naturally occurring, seasonal IgE-mediated skin allergy in horses (34–42) caused by salivary allergens from biting *Culicoides* midges. Horses with *Culicoides* hypersensitivity develop dermatitis, pruritis, and skin lesions in response to bites from *Culicoides* midges and exposure to *Culicoides* salivary protein allergens (40, 43–46). *Culicoides* hypersensitivity starts as an acute allergic inflammatory disease in early summer when the midges appear in the environment, develops into chronic allergic dermatitis with ongoing allergen exposure, and resolves in late fall when the midges are not present anymore. Allergic horses serve as a unique model for

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Abbreviations used in this article: CHO, Chinese hamster ovary; FPLC, fast protein liquid chromatography; FSC, forward scatter; MHC II, MHC class II; RAO, recurrent airway obstruction; SSC, side scatter.

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human allergy by sharing a similar immune cell profile with allergic humans, including rare peripheral IgE-binding monocytes and basophils (47–51). We use the horse as a model for IgE-mediated allergy with the ability to control for one environment, and similar allergen exposure, nutrition, and treatment, which remain highly variable in human populations (52). These controlled variables, along with the ability to collect large blood volumes, allow for the study of rare immune cells that are otherwise difficult to identify and resolve.

In this article, we identified IL-8–producing monocytes, including IgE-binding monocytes, and basophils in equine peripheral blood. This is consistent with human IL-8–producing cells and supports the horse as a relevant model for human immune conditions. LPS and mitogens are the primary stimulators of IL-8 in humans, but IgE-mediated IL-8 may also have a yet underappreciated role in the allergic mechanism. The specific IgE-mediated intracellular pathways for IL-8 induction have been partially explored in human mast cells (15, 53) but not in IgE-binding monocytes. This is, to our knowledge, the first article comparing IL-8 induction by IgE-binding monocytes between allergic and nonallergic individuals and identifying a novel role for IgE-mediated IL-8 production by monocytes during allergy.

Materials and Methods

Recombinant equine IL-8

RNA was extracted from horse PBMC and transcribed into cDNA as previously described (54, 55). Equine IL-8 cDNA was first amplified using primers flanking the entire coding sequence (GenBank accession number NM_001083951; bases 66 to 428; forward primer, 5'-ACCAGAAAGACAGCATCTCGT-3'; reverse primer, AGAACGCGTTGTTGGTGC-3'). The entire equine IL-8 gene was then cloned into the mammalian expression vector IL-4/pcDNA3.1, as previously described (54), using primers containing restriction enzyme sites (forward primer with *Bam*H I restriction site 5'-AAGGATCCTGCGTTGTATCAAGAAT-TACTGCAGAG-3'; reverse primer with *Hind* III restriction site: 5'-AAATT-TAAGCTTTCATGGATTTTGCCCTCAGTCTC-3'). IL-4 serves as a tag in this expression system.

To express rIL-4/IL-8 in mammalian cells, a total of 20 µg of IL-4/IL-8/pcDNA3.1 expression vector was transfected into Chinese hamster ovary (ExpiCHO-S) cells (Thermo Fisher Scientific Waltham, MA). Cell-free, serum-free supernatants were collected after 6 d and purified over an anti-IL-4 affinity column. The column was prepared by coupling IL-4 mAb 13G7 (56) covalently to a HiTrap NHS-activated HP column (GE Healthcare, Piscataway, NJ), as previously described. IL-8 purification was performed on an ÄKTA fast protein liquid chromatography (FPLC) instrument (GE Healthcare). The correct protein size was confirmed on a reducing 12% polyacrylamide gel and a PageRuler Prestained Protein Ladder (10–180 kDa, Thermo Fisher Scientific). The gel was stained with Coomassie Brilliant Blue dye (Sigma-Aldrich, St. Louis, MO). Previous development of anti-equine mAbs describe these methods in more detail (54, 55, 57, 58).

mAb generation

One C57BL/6J mouse (The Jackson Laboratory, Bar Harbor, ME) was immunized with 50 µg of purified rIL-4/IL-8 and 100 µl of GERBU Adjuvant (GERBU Biotechnik, Heidelberg, Germany) i.p. per day on days 0, 14, 21, 36, 61, 141, and 171. On days 218, 219, and 220, injections of 50 µg of rIL-4/IL-8 were given without adjuvant, and the mouse was euthanized on day 221 for collection of splenocytes, as previously described (57). Splenocytes were fused with murine X63-Ag8.653 myeloma cells (59) following the previously described cell fusion protocol (54, 57).

Hybridoma supernatants were initially tested for IL-8 specificity by ELISA. First, wells were coated overnight with polyclonal anti-IL-4 Ab (R&D Systems, Minneapolis, MN) at 4°C in carbonate coating buffer (1 M NaHCO₃, 1 M Na₂CO₃, [pH 9.6]). Wells were washed with phosphate buffered saline (0.05% Tween 20), and wells were incubated with rIL-4/IL-8 ExpiCHO-S cell-free supernatant for 1 h at room temperature. This was followed by another wash and incubation of wells with hybridoma clone cell-free supernatants. Binding of mAbs was then detected using a peroxidase-conjugated goat anti-mouse IgG(H+L) Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Next, IL-8 ELISA-positive clones were tested on similar ELISAs against equine rIL-8/IgG1 and rIL-4/IgG1. One mAb clone, 321-2, recognizing IL-8 but not IL-4 or IgG1 by ELISA, was obtained and

isotyped as mouse IgG1 (Sigma-Aldrich, St. Louis, MO). IL-8 mAb 321-2 was adapted to serum-free medium (Hybridoma-SFM; Life Technologies, Thermo Fisher Scientific), and the mAb was purified from cell-free supernatant using a HiTrap Protein G HP column (GE Healthcare) by FPLC. A non-IL-8 binding mAb from the same fusion, clone 379-1, was also adapted to serum-free medium, purified, and used as a mouse IgG1 isotype control. Both purified mAbs were conjugated to Alexa Fluor 488 or 647 (Thermo Fisher Scientific) following manufacturer's instructions.

Transfection of Chinese hamster ovary K1 cells

To confirm that IL-8 mAb 321-2 was specific for rIL-8, adherent Chinese hamster ovary (CHO)–K1 cells (Thermo Fisher Scientific) were transiently transfected with rIL-8/IgG1, rIL-4/IL-8, or rIL-4/IgG1 sequences cloned into IL-4/pcDNA3.1 or IgG1/pcDNA3.1 expression vectors using GenePORTER 2 Transfection Reagent (Gene Therapy Systems, San Diego, CA). Cells were harvested 24 h after transfection, fixed in 2% (v/v) formaldehyde (Sigma-Aldrich), and stored at 4°C until they were stained and analyzed by flow cytometric analysis.

Horses, allergy groups, and blood sampling

Heparinized blood was obtained from the jugular vein of six mares (8–16 y, median 14 y), one gelding (9 y), and one stallion (8 y) using the BD Vacutainer system (Becton Dickinson, Franklin Lakes, NJ). For comparison of IL-8 production in allergic and nonallergic horses, blood was collected from horses with *Culicoides* hypersensitivity ($n = 7$) and from nonallergic horses ($n = 7$). Allergic horses consisted of five mares, one gelding, and one stallion, and nonallergic horses consisted of five mares and two geldings. All horses were Icelandic horses.

Culicoides hypersensitivity is a naturally occurring, seasonal recurrent allergic disease. Clinical signs of allergy only develop during summer exposure to *Culicoides* allergen, and symptoms resolve during winter months. Clinical allergy scores were assigned based on a scoring system, in which scores ≥ 3 were considered allergic, as previously described (60). All allergic horses exhibited clinical signs of *Culicoides* hypersensitivity (pruritis, alopecia, and skin irritation) for several months during the summer and while exposed to *Culicoides* allergen in the year of this study, as well as at least 1 y prior to the study. Allergy symptoms in allergic horses were resolved in winter months, when *Culicoides* were absent from the environment. Nonallergic horses never experienced clinical signs at any timepoint, with or without *Culicoides* allergen exposure (Table II).

Culicoides-specific hypersensitivity was further confirmed by immediate skin reactions to intradermal skin testing with *Culicoides* whole-body extract (Stallergenes Greer, Cambridge, MA.). Intradermal skin testing with saline and histamine served as controls, as previously described (47, 49). Nonallergic horses did not react to intradermal *Culicoides* whole-body extract injections. All horses were housed together in the same environment at the Animal Research Facilities at Cornell University. They lived 24 h/7 d on pastures with a run-in shed and free access to water and salt blocks. Horses were all on the same diet (grass hay during the winter and grass during the summer) and were annually vaccinated on the same days against rabies (January), tetanus (June), and dewormed (December). During the sampling period in February, when *Culicoides* were absent from the environment, none of the horses displayed clinical signs of allergy.

All animal procedures were carried out in accordance with the recommendation in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee at Cornell University (protocol number 2011-0011). The study also followed the *Guide for Care and Use of Animals in Agricultural Research and Teaching*. All horses survived the study and were kept as research horses at Cornell University afterward.

PBMC isolation and stimulation

Equine erythrocytes spontaneously sediment and separate from the leukocyte-rich plasma 30–60 min following collection into heparin. Equine PBMC were purified from the leukocyte-rich, erythrocyte-depleted plasma by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare) and immediately cultured in vitro in DMEM supplemented with 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 50 µM 2-ME, 50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin (Thermo Fisher Scientific Waltham, MA), and 10% FCS (Atlanta Biologicals, Flowery Branch, GA). A total of 3×10^6 cells/ml were cultured at 37°C in 14 ml of polypropylene round-bottom tubes (Falcon; Corning, NY) that had been precoated with DMEM (10% FBS) overnight to minimize monocyte/macrophage cell adhesion. The medium was supplemented with either 25 ng/ml PMA and 1 µM ionomycin (PMA/ionomycin), or 1 µg/ml PHA, or 20 ng/ml *Escherichia coli* 055:B5 LPS, or 2.5 µg/ml PWM (all from Sigma-Aldrich, St. Louis, MO), or 1 µg/ml IgE mAb 134 (57), or 1 µg/ml IgE mAb 176 (57). PBMC

in medium alone were used as an unstimulated control. IgE mAb 134 cross-links IgE-bound IgE molecules on cell surfaces, resulting in IgE-mediated stimulation (47–51). IgE mAb 176 does not cross-link the IgE/IgE and served as an isotype stimulation control (47–51). All *in vitro* stimulations were performed in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich, St. Louis, MO). Time titration experiments compared IL-8 production after *in vitro* stimulation for 0.5, 1, 2, 4, 6 h, and overnight (19–21 h). All phenotyping experiments were performed after 2 h of *in vitro* stimulation. Following stimulation, PBMC were fixed in 2% (v/v) formaldehyde (Sigma-Aldrich) for intracellular Ab staining and flow cytometric analysis. For viability analysis, cells were incubated with fixable LIVE/DEAD viability dye (Thermo Fisher Scientific) before fixing, according to manufacturer's instructions. Live cells are negative for the viability dye.

Intracellular Ab staining and IL-8 detection by flow cytometry

For initial hybridoma clone screening by flow cytometry, fixed transfected CHO cells were incubated with IL-8 hybridoma supernatants in saponin buffer (0.5% saponin, 0.5% BSA, 0.02% NaN₃ in PBS), followed by incubation with goat anti-mouse (H+L) F(ab')₂ Ab conjugated to Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, West Grove, PA). Transfection efficiency was measured with equine IL-4 mAb 13G7 (56) or equine IgG1 mAb CVS45 (61). To confirm specificity of IL-8 mAb clone 321-2, CHO cells were transfected with different IL-4 and IgG1 expression vectors, as outlined above. Transfectants were fixed and incubated with purified IL-8 mAb 321-2 directly conjugated to Alexa Fluor 647. A total of 10,000 events per sample were recorded and analyzed in a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed on FlowJo version 10.4 (FlowJo, Ashland, OR).

Abcs were conjugated to Alexa Fluor 488 or 647 (Thermo Fisher Scientific), biotin, or PE, following manufacturer's instructions. Fixed equine PBMC were stained intracellularly with different master mixes (Table I) in saponin buffer (0.5% saponin, 0.5% BSA, 0.02% NaN₃ in PBS). Cells were washed and then incubated with streptavidin-PE (Jackson ImmunoResearch Laboratories) or streptavidin-PerCP-cyanin5.5 (BioLegend) in PBS-BSA (0.5% BSA, 0.02% azide; Sigma-Aldrich, St. Louis, MO) to label biotinylated mAbs. A total of 50,000 events per sample were recorded for master mixes 1 and 2; 100,000 events per sample were recorded for master mixes 3 and 6–12; and 200,000 events per sample were recorded for master mixes 4 and 5. Cells were recorded and analyzed, as described above. Compensation was calculated manually for 2 and 3 color master mixes 3 and master mixes 6–12 using input from single-stained ArC Amine Reactive Compensation Beads (Thermo Fisher Scientific) and cells for master mix 1; UltraComp beads (Thermo Fisher Scientific) were used for master mix 4. Master mix 4 was used to quantify MHC class II (MHCII), CD14, and IgE expression on stimulated PBMC and to compare IL-8⁺ basophils and IL-8⁺ IgE-binding monocytes between horse groups.

Statistical analysis

Data sets were tested by Shapiro–Wilk tests, and most sets were not normally distributed. In some comparisons, one sample group was normally distributed and the other was not. In these cases, both sample sets were analyzed using non-Gaussian statistical tests. IL-8 production in response to different stimuli and incubation times was graphed as mean with SEM. Comparisons between allergic and nonallergic horse groups (percentages of IL-8⁺ cells, basophils, IgE-binding monocytes, IL-8⁺ basophils, and IL-8⁺ IgE-binding monocytes, all out of total PBMC) were graphed as individual horse values and medians for each group. Allergic and nonallergic groups were compared for each stimulation using an unpaired nonparametric Mann–Whitney *U* test. All *p* values <0.05 were considered statistically significant. Analysis was performed with GraphPad Prism software version 8 (GraphPad Software, La Jolla, CA). All raw data are included for reference (Supplemental Table I).

Results

Specificity of IL-8 mAb 321-2

Using hybridoma technology, a mAb against equine IL-8 was generated. The size of purified recombinant rIL-4/IL-8 used for mouse immunizations was determined as ~30 kDa in a Coomassie gel (Fig. 1A). The calculated molecular mass of rIL-4/IL-8 was 24 kDa, not including glycosylation weight. Human IL-8 is not glycosylated (62), and equine IL-8 is expected to be similar. There were two bands of around 26 and 30 kDa that were likely due to different IL-4 glycosylation patterns, as observed previously (54, 63).

IL-8 mAb 321-2 was obtained after screening all hybridoma supernatants against rIL-4/IL-8 by ELISA and subsequent testing of all positive supernatants against rIL-8/IgG1 and rIL-4/IgG1. Specificity of the IL-8 mAb 321-2 for its target protein was further confirmed by intracellular staining and flow cytometric analysis of CHO cells transfected with different equine recombinant proteins. CHO cells were first gated by forward (FSC) and side scatter (SSC) characteristics (Fig. 1B). IL-8 mAb 321-2 and IL-4 mAb but not IgG1 mAb recognized a similar population of IL-8⁺ and IL-4⁺ cells, respectively, in an equine rIL-4/IL-8 transfectant (Fig. 1C). Similarly, IL-8 mAb 321-2 and IgG1 mAb but not IL-4 mAb labeled the rIL-8/IgG1 transfectant (Fig. 1D). In contrast, IL-8 mAb 321-2 did not detect any cells in rIL-4/IgG1-transfected (Fig. 1E) CHO cells. However, both IL-4 and IgG1 mAbs labeled the rIL-4/IgG1-transfected cells. IL-4 and IgG1 mAb stainings served to confirm successful CHO cell transfections. Based on these results, anti-equine IL-8 mAb 321-2 was specific for its recombinant equine IL-8 target.

IL-8 mAb 321-2 detection of native equine IL-8

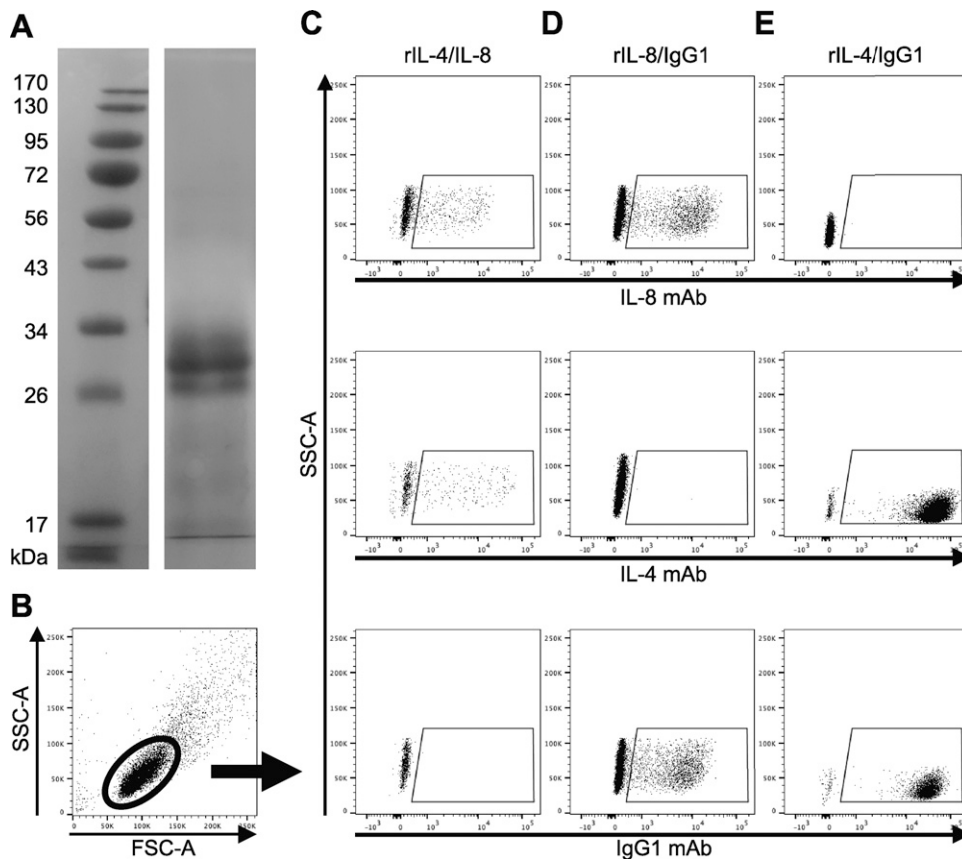
IL-8 mAb 321-2 was next used to identify native IL-8 production in equine PBMC by intracellular staining and flow cytometry. PBMC were cultured with different stimuli to provoke IL-8 production. A morphology gate was first set on PBMC using FSC and SSC characteristics (Fig. 2A). A mouse IgG1 isotype control was included in the staining and did not detect any PBMC following 4 h of *in vitro* culture with PHA (Fig. 2B) or any of the other timepoints or stimuli (Supplemental Fig. 1A). IL-8 production by culturing PBMC in medium alone was not detectable prior to 4 h of stimulation. Spontaneous IL-8 production in medium alone occurred in live cells after 4 h and all longer timepoints (Supplemental Fig. 2). After PHA stimulation, percentages and kinetics of IL-8-producing cells were similar to IL-8 detected in the medium control (Fig. 2C, 2D). Native IL-8 was produced after 2 h of culture with PMA/ionomycin, LPS, or PWM and afterward until at least 20 h poststimulation (Fig. 2C, 2D). Stimulation patterns were consistent in all horses tested with the highest percentages of IL-8-producing cells observed after 2 h of LPS stimulation (median 3.0%, range 2.8–3.3%; Fig. 2D). Together, these data supported that IL-8 mAb 321-2 detected native equine IL-8.

IL-8 production by peripheral blood monocytes

We next determined which cells in peripheral blood produced IL-8 in response to 2 h of LPS stimulation. We used different Ab combinations to identify T cells, B cells, and monocytes, together with intracellular staining for IL-8⁺ cells (Table I), and analyzed the cells by flow cytometry. In response to LPS stimulation, the majority of IL-8⁺ PBMC expressed CD14 (Fig. 3A) and MHCII (Fig. 3B), whereas they lacked CD16 (Supplemental Fig. 3A), and were considered CD14⁺/MHCII⁺/CD16[−] monocytes. In addition, a subset of IL-8⁺ monocytes were also IgE⁺ (Fig. 3C) and were considered IgE-binding monocytes.

The IL-8⁺ monocyte identity was further confirmed by the absence of several other phenotypic markers. All IL-8⁺ cells were CD8[−]/IgM[−]/IgG1[−]/IgG4[−] (Supplemental Fig. 3B–E). Although most IL-8⁺ cells also expressed CD4 (Supplemental Fig. 3F), additional staining with T cell CD3 and monocyte CD14 markers confirmed that IL-8⁺/CD4⁺ cells were also CD3[−]/CD14⁺ (Supplemental Fig. 3G), which we considered CD4⁺ monocytes. Likewise, a smaller subset of IL-8⁺ cells were CD5⁺/CD3[−]/CD14⁺ (Supplemental Fig. 3H, 3I) and were

FIGURE 1. IL-8 mAb 321-2 specifically detects recombinant equine IL-8 by flow cytometry. CHO cells were transfected with different expression vectors for equine fusion proteins. Cells were harvested, fixed, and incubated with IL-8 mAb 321-2 or with mAbs against equine IgG1 or IL-4 and analyzed by flow cytometry. **(A)** Recombinant rIL-4/IL-8 from CHO cell supernatant was purified using an anti-IL-4 affinity column by FPLC. A 10–180 kDa protein ladder (left) and an aliquot of the purified rIL-4/IL-8 protein (right) were separated on a reducing 12% polyacrylamide gel and stained with Coomassie Brilliant Blue dye. **(B)** CHO cells were gated by size on doublet-excluded cells. Cells transfected with **(C)** rIL-8/IgG1, **(D)** rIL-4/IL-8, or **(E)** rIL-4/IgG1 and were stained with IL-8 mAb 321-2 (top), IL-4 mAb (middle), or IgG1 mAb (bottom).



considered CD5⁺ monocytes. Together, these data supported that LPS stimulates IL-8 production by different CD14⁺/MHCII⁺/CD16⁺ monocyte subpopulations, including IgE-binding monocytes. This validated the horse as a model for IL-8 production in humans.

IL-8 production in response to IgE cross-linking

Human IgE-binding monocytes produce IL-8 after IgE cross-linking (19). We asked whether IgE cross-linking could also stimulate IL-8 production in horses. We incubated PBMC with cross-linking IgE mAb 134, which stimulates cells through the IgER (47–51, 57). IL-8 production in response to IgE cross-linking was detectable after 1 h of stimulation, peaked after 2 h (median 2.3%, range 0.8–4.4% IL-8⁺ cells out of total PBMC; Fig. 4A, 4B), and was still detectable after 6 h. A mouse IgG1 isotype control was included in the staining and did not detect any PBMC after stimulation with IgE mAb 134 for 2 h (Fig. 4C) or any other timepoint (Supplemental Fig. 1B). As an additional control, PBMC incubated with a non-cross-linking IgE mAb 176 did not produce IL-8 after 2 h (Fig. 4D) and were also not detected with isotype control staining (Supplemental Fig. 1B). Incubations longer than 4 h with IgE mAb 176 induced delayed IL-8 production in PBMC (Supplemental Fig. 1C). These data confirmed that IgE cross-linking rapidly stimulates IL-8 production and suggested that IL-8 is likely involved in the early IgE-mediated recruitment of inflammatory cells.

Comparison of IL-8 production in allergic and nonallergic horses

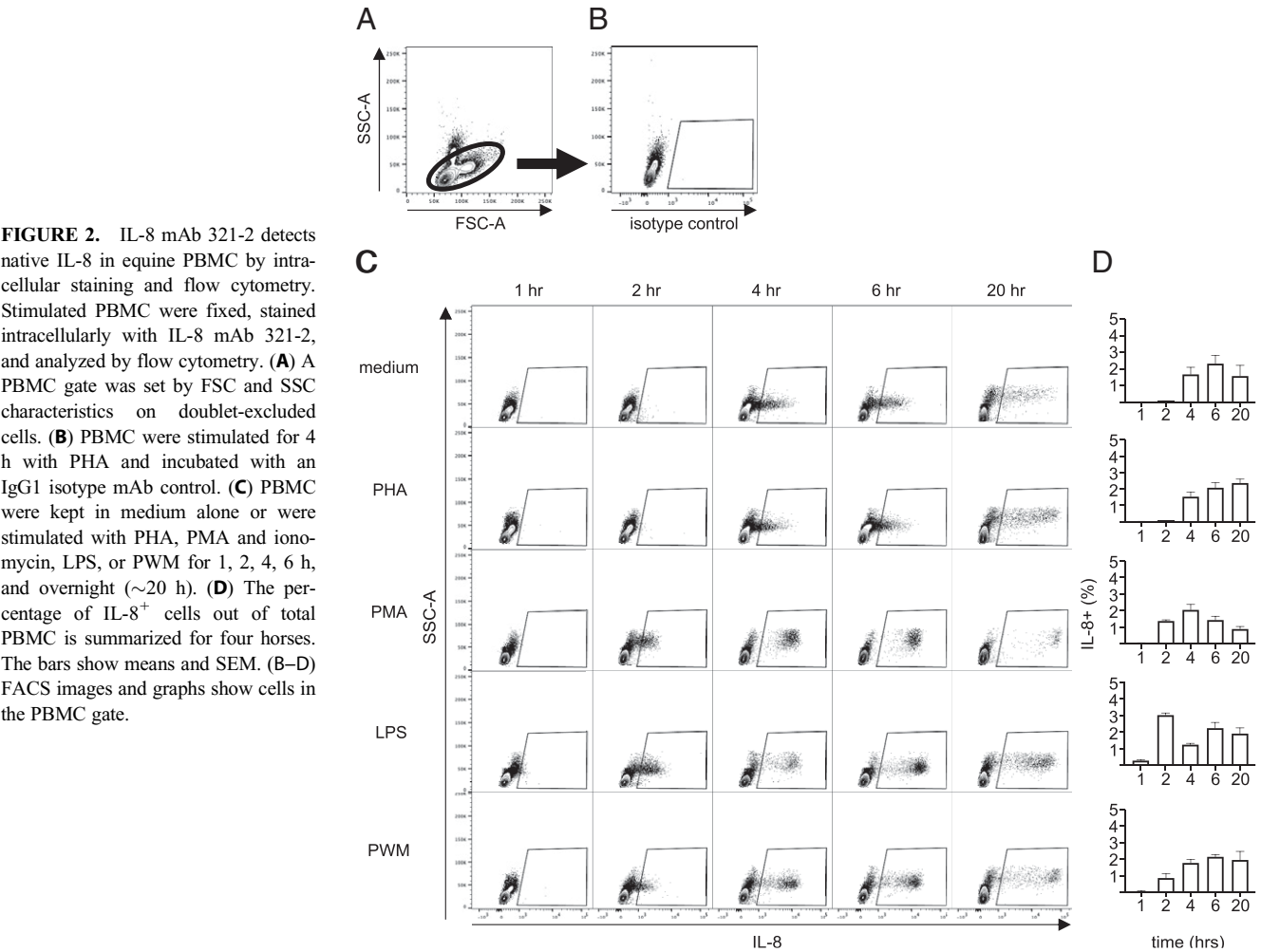
To explore whether allergic horses have higher percentages of IL-8-producing cells in response to IgE cross-linking, we compared PBMC from allergic horses with *Culicoides* hypersensitivity ($n = 7$)

with those from healthy nonallergic control horses ($n = 7$) (Table II) after stimulation with IgE mAb 134 for 2 h. PBMC from allergic horses had a trend toward increased IL-8⁺ cells (median 1.5%, range 0.6–1.7%) compared with nonallergic horses (median 0.9%, range 0.6–2.0%) in response to IgE cross-linking. (Fig. 5). There was no IL-8 production by cells cultured in medium alone, supporting that spontaneous release of IL-8 in culture had not begun.

PBMC from both horse groups were also stimulated with PMA and ionomycin or LPS (Fig. 5). PMA/ionomycin stimulated 0.4–1.5% IL-8⁺ PBMC, which was overall slightly lower than the percentage of IL-8⁺ PBMC following IgE cross-linking (0.7–3.1% IL-8⁺ PBMC). After PMA/ionomycin stimulation, and similar to IgE cross-linking, cells from allergic horses showed a trend toward increased percentages of IL-8⁺ PBMC that was not statistically significant between allergic and nonallergic horses. In response to LPS stimulation, horses with *Culicoides* hypersensitivity had a significantly higher percentage of IL-8⁺ PBMC (median 2.4%, range 1.2–3.1%) compared with nonallergic horses (median 1.1%, range 0.7–1.4%). These data demonstrated that IL-8 is more readily produced by PBMC from allergic horses compared with healthy horses, especially after LPS stimulation.

IL-8 production by IgE-binding monocytes and basophils after IgE cross-linking

We next characterized which cell types produced IL-8 upon IgE cross-linking. Similar to humans, there are two distinct cell populations in equine PBMC that bind IgE through the FcεRI and can be stimulated by IgE cross-linking: basophils, which are IgE⁺/MHCII^{low}/CD14⁺ (Fig. 6A, 6B) (48), and IgE-binding monocytes, which are characterized by IgE⁺/MHCII^{high}/CD14^{low} (Fig. 6A, 6B) (51). The majority of IgE⁺/MHCII^{high} cells are IgE-binding monocytes.



We stimulated PBMC with IgE mAb 134 for 2 h and analyzed IL-8 expression by flow cytometry. IL-8⁺/IgE⁺ cells were gated within the basophil (Fig. 6C) and IgE-binding monocyte gates (Fig. 6D). The

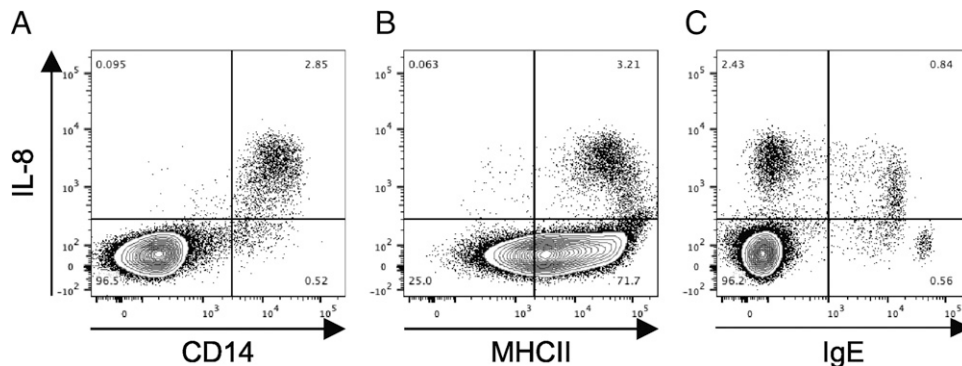
percentage of IL-8⁺ basophils and IL-8⁺ IgE-binding monocytes out of total PBMC were compared between allergic and nonallergic horse groups (Fig. 6E, 6F). The percentage of IL-8⁺ basophils was low

Table I. Ab master mixes for phenotyping of IL-8-producing cells by flow cytometry

Master Mix	Alexa Fluor 647	Alexa Fluor 488	PE ^a	PerCP-Cyanin 5.5 ^a	Near-IR (allophycocyanin-Cy7)
1	Equine IL-8 ^b	N/A	N/A	N/A	Live/Dead
2	Equine IL-8 ^b	N/A	N/A	N/A	N/A
3	Equine IL-8 ^b	Equine CD16 ^c	Equine CD14 ^d	N/A	N/A
4	Equine IL-8 ^b	Equine IgE ^e	Equine MHCII ^f	Equine CD14 ^d	N/A
5	Equine IL-8 ^b	Equine IgE ^e	Equine MHCII ^f	N/A	N/A
6	Equine IL-8 ^b	Equine IgM ^g	Equine CD5 ^h	N/A	N/A
7	Equine IL-8 ^b	Equine IgG1 ⁱ	Equine IgG4 ^j	N/A	N/A
8	Equine IL-8 ^b	Equine CD4 ^k	Equine CD8 ^l	N/A	N/A
9	Equine IL-8 ^b	Equine CD14 ^d	Equine CD4 ^k	N/A	N/A
10	Equine IL-8 ^b	Equine CD14 ^d	Equine CD5 ^h	N/A	N/A
11	Equine CD3 b ^m	Equine IL-8 ^b	Equine CD4 ^k	N/A	N/A
12	Equine CD3 b ^m	Equine IL-8 ^b	Equine CD5 ^h	N/A	N/A

^amAb was biotinylated and fluorescently labeled with streptavidin-PE or streptavidin-PerCP-Cyanin 5.5. MHCII mAb in master mix 4 was directly coupled to PE.
^bClone 321-2.
^cClone 9G5 (63).
^dClone 105 (66).
^eClone 176 (57).
^fClone cz11 (65).
^gClone 1-22 (64).
^hClone CVS5 (67).
ⁱClone CVS45 (61).
^jClone CVS39 (61).
^kClone HB61A (65).
^lClone CVS8 (67).
^mClone F6G (62).

FIGURE 3. Peripheral blood monocytes are the main IL-8 producers in response to LPS stimulation. PBMC were stimulated for 2 h with LPS and stained intracellularly with IL-8 mAb 321-2 and with mAbs for different cell surface proteins: **(A)** CD14 and IL-8, **(B)** MHCII and IL-8, **(C)** and IgE and IL-8. All FACS images show cells in the PBMC gate and are representative images from one horse. The experimental procedure was repeated with PBMC from four horses.



overall and similar in allergic (median 0.005%, range 0.0–0.01% out of PBMC) and nonallergic horses (median 0.001%, range 0.000–0.012% out of total PBMC; Fig. 6E). In contrast, there were significantly more IL-8⁺ IgE-binding monocytes in allergic horses (median 0.20%, range 0.10–0.27% out of PBMC) compared with nonallergic horses (median 0.11%, range 0.09–0.18% out of PBMC; Fig. 6F). The percentages of total basophils and IgE-binding monocytes out of PBMC following IgE cross-linking were similar between horse groups (Fig. 6G). Together, these data showed that IgE-binding monocytes in allergic horses have an enhanced capacity for IL-8 production upon IgE cross-linking compared with healthy horses, supporting a role for these cells in cell recruitment during allergic inflammation.

Discussion

IL-8 is critically involved in the innate immune response, recruitment of immune cells, and elimination of pathogens. However, it can also majorly contribute to inappropriate or excessive inflammation in diseases such as asthma, atherosclerosis, chronic obstructive pulmonary disease, inflammatory bowel disease, and the promotion of angiogenesis and tumor growth (64). Unlike mice, horses share many of these diseases with humans and thus represent a heterogeneous large animal model of naturally occurring inflammatory disorders (25). Equine IL-8 is essential in the recruitment of neutrophils and disease clearance of pathogens (31, 65) and also promotes inflammatory diseases such as RAO or equine asthma (27–29, 31). In this study, we observed elevated IL-

8 production by IgE-binding monocytes in allergic individuals, supporting a mechanistic role for IL-8 in allergy.

Horses not only present a unique model to study immune mechanisms in naturally occurring inflammatory diseases, but their environment can also be controlled in experimental settings and thereby eliminate many of the confounders of human clinical studies. In addition, large volumes of blood can be routinely collected, allowing for repeated study of rare peripheral cells, such as IgE-binding monocytes. In this study, we investigated horses with an IgE-mediated skin allergy called *Culicoides* hypersensitivity to explore IL-8 production in innate immune cells in the peripheral blood. *Culicoides* hypersensitivity is a seasonal disease and mechanistically similar to IgE-mediated allergies in human patients (40, 45, 47–51). Allergic and healthy horses all lived together in the same environment for many years. Therefore, these horses were all exposed to similar *Culicoides* species and *Culicoides* allergens, similar frequency of bites leading to allergen exposure, and similar seasonal timing of allergen exposure and clinical signs of allergy. In addition, other parameters, such as nutrition, vaccination, and deworming were identical for all horses. Timing and frequency of allergen exposure is a critical variable that cannot be controlled for in human populations (66).

We first developed a new, to our knowledge, anti-equine IL-8 mAb (clone 321-2) for detection and study of equine IL-8 and confirmed that equine IL-8 was predominantly produced by peripheral blood CD14⁺/MHCII⁺/CD16⁻ monocytes but not B or T cells after LPS stimulation via CD14 binding and activation of the TLR4 pathway.

FIGURE 4. IgE cross-linking stimulates rapid IL-8 production. PBMC were stimulated with cross-linking IgE mAb 134, fixed and analyzed for intracellular IL-8 expression by flow cytometry. **(A)** PBMC were stimulated for 30 min, 1, 2, 4, and 6 h. **(B)** The percentage of IL-8⁺ cells out of total PBMC after IgE cross-linking is summarized using four different horses. **(C)** Anti-IgE 134 stimulated PBMC (2 h) were also analyzed for intracellular staining with an IgG1 isotype mAb control. **(D)** PBMC were also stimulated for 2 h with a non-cross-linking control IgE mAb 176, fixed, and analyzed for intracellular staining with IL-8 mAb 321-2. All FACS images show PBMC-gated cells. The experimental procedure was repeated with PBMC from four horses.

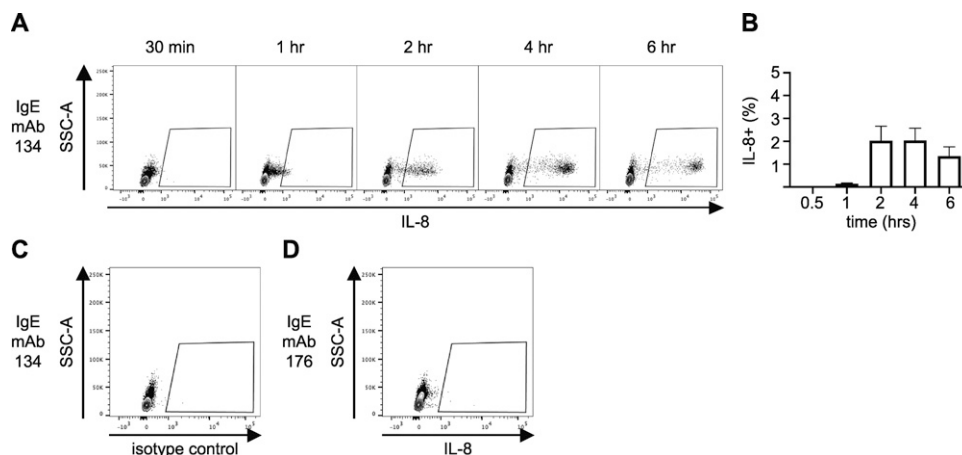


Table II. Clinical allergy scores of horses with *Culicoides* hypersensitivity and clinically healthy control horses living in the same environment with seasonal *Culicoides* exposure

	Nonallergic Horses (n = 7)	Allergic Horses (n = 7)
Age, median (range) in y	8 (7–9)	15 (8–17)
Clinical score, median (range)		
With <i>Culicoides</i> exposure ^a	0 (0–2)	5 (2–9)
Without <i>Culicoides</i> exposure ^b	0 (0–0)	0.8 (0–2)

^a*Culicoides* exposure and clinical signs of allergy occurred from June to October 2019.

^bNo *Culicoides* exposure. Months were from November 2019 to February 2020. Horses did not show clinical signs of allergy during this time. In the summer of 2020, allergic horses developed clinical allergy again.

This finding is consistent with previous IL-8 mRNA studies in humans and horses (8, 29, 32, 67) and served to validate our model. We also confirmed that IgE cross-linking induced IL-8 production in equine basophils and IgE-binding monocytes, supporting an FcεRI-mediated activation pathway in both cell types. IL-8 production by basophils and IgE-binding monocytes has previously been reported in human studies (5–7, 16, 17, 19, 20) and further supports the use of the well-controlled horse model as a comparative approach for studying IL-8 in the context of IgE-mediated allergy.

There are two variants of FcεRI. In humans and horses, basophils and mast cells express a tetrameric αβγ₂ FcεRI receptor, whereas IgE-binding monocytes express a trimeric αγ₂ FcεRI receptor that lacks the β-chain (47–51, 68). The α-chain binds IgE and induces intracellular signaling through both the β and γ subunits, which are transmembrane proteins containing ITAMs. In humans, the γ-chain ITAM recruits Syk kinase to initiate a signaling cascade (69). The β-chain amplifies this response through recruitment of Lyn kinase and subsequent recruitment and phosphorylation of Syk kinase (69, 70). A truncated form of the β-chain has been identified in human mast cells and is thought to inhibit IL-8 production and mast cell degranulation (71). This truncated FcεRI β-chain has not been identified in horses. We detected IL-8 production after IgE cross-linking of basophils, supporting that equine IL-8 is activated in basophils after FcεRI stimulation.

As mentioned above, IL-8 is predominantly produced by monocytes in humans and horses. Monocytes in both species are characterized by expression of CD14 and CD16 into classical, intermediate, and nonclassical monocytes (72–75). Recent human studies have demonstrated the vast diversity of monocyte subpopulations and respective functional abilities, including proinflammatory cell recruitment, tissue repair, and mobilization of immune cell precursors (76, 77). The balance of monocyte types within an individual has also

been correlated to different inflammatory diseases (78). It was therefore expected that IL-8⁺ CD14⁺ monocytes comprise several specific subpopulations of equine monocytes.

One of these monocyte subpopulations is IgE-binding monocytes, which we have recently described in horses as CD14^{low}/MHCII^{high}/IgE⁺ cells (51). Equine IgE-binding monocytes are remarkably similar to the corresponding population in humans (19, 20, 79–81). We previously showed that equine IgE-binding monocytes are rare and comprise ~6% of total equine peripheral blood monocytes. After LPS stimulation, almost 25% of IL-8⁺ monocytes were also IgE⁺. This disproportionately high frequency of IgE-binding monocytes suggested that IgE-binding monocytes are substantially responsible for IL-8 production, independent of IgER signaling. We further explored the mechanism of IL-8 production in IgE-binding monocytes after IgE cross-linking, which are also known to exhibit unique IgE-mediated functional responses (51).

Human IgE-binding monocytes are known to produce IL-1β, IL-6, IL-8, IL-10, and TNF-α after IgE cross-linking (19, 20). Similarly, we have confirmed that equine IgE-binding monocytes also produce IL-10 (51) and IL-8 in response to IgE cross-linking. Interestingly, we found that not all IgE-binding monocytes produced IgE-mediated IL-8. We suggest two possible mechanisms to explain the production of proinflammatory IL-8 in allergic horses by some but not all IgE-binding monocytes (1): IgE-binding monocytes can be polarized to either express IL-8 or IL-10, depending on the local environment and milieu of IgE-mediated cytokines and chemokines, or (2) IgE-binding monocytes comprise different functional subpopulations in allergic and healthy individuals. Further work is necessary to understand the nuances of the IgE-binding monocyte function in the context of IgE-mediated activation and allergic diseases.

To the best of our knowledge, this is the first study to compare IL-8 production between allergic and healthy individuals after IgE stimulation by basophils and IgE-binding monocytes. IL-8⁺ basophils have been compared in allergic humans but only after TLR stimulation (16). In this article, we show that allergic individuals have increased percentages of peripheral IL-8⁺ IgE-binding monocytes compared with healthy controls upon IgE cross-linking. This suggests that IgE-binding monocytes can increase allergic inflammation by recruiting inflammatory neutrophils and basophils via IL-8. Basophil recruitment by IgE-binding monocytes could further enhance the severity of an inflammatory response. The development of the new IL-8 mAb will allow further examination of IgE-mediated IL-8 production in cells isolated from local sites of allergic inflammation, such as skin lesions.

IgE-mediated skin allergies, such as *Culicoides* hypersensitivity, are initiated rapidly because of local mast cell degranulation, resulting in acute allergic inflammation (47, 50, 82), and can persist as a chronic inflammatory response if the individual is continuously exposed to allergen. Allergic horses with *Culicoides* hypersensitivity only develop clinical signs of allergy during the summer, when the midges are regularly feeding and the horses are exposed to *Culicoides* salivary allergens. The experiments reported in this study took place during the winter, when *Culicoides* were not present in

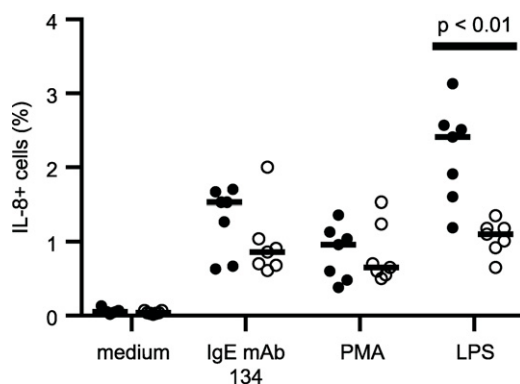
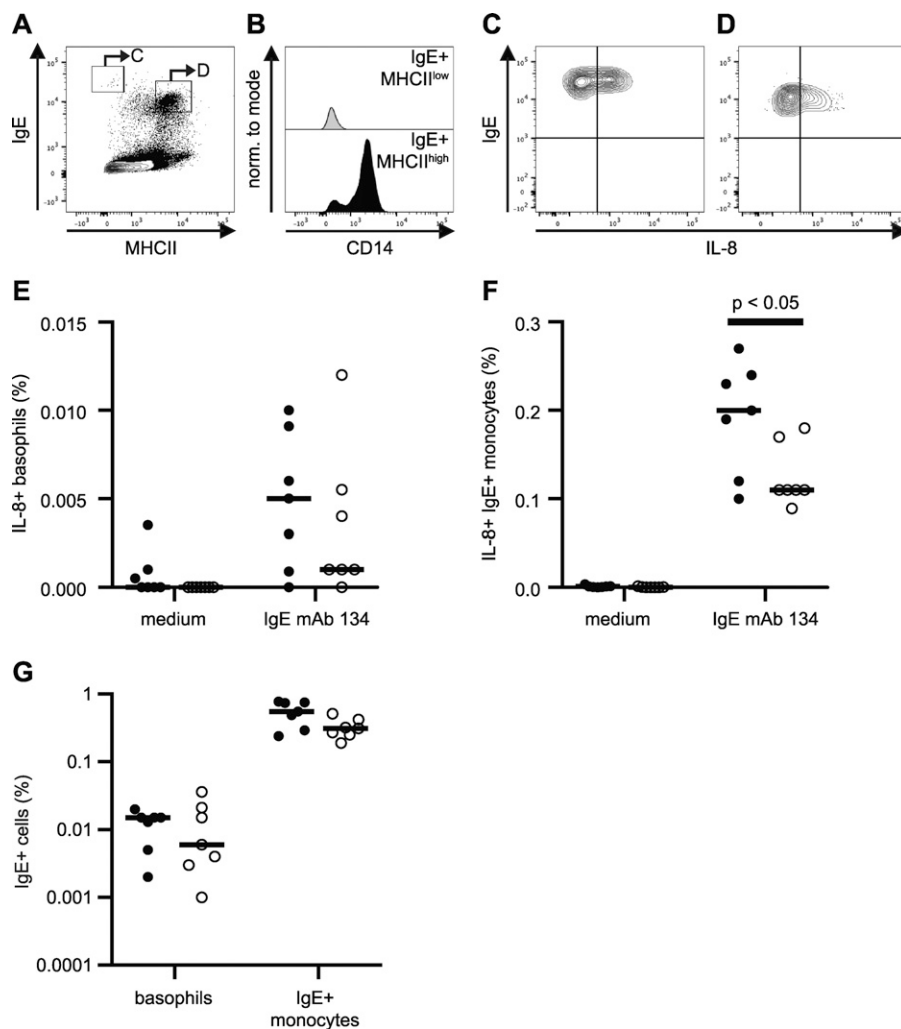


FIGURE 5. IL-8 production in allergic and nonallergic horses. PBMC were stimulated for 2 h with medium alone, cross-linking IgE mAb 134, PMA/ionomycin, or LPS. Cells were fixed, stained intracellularly with IL-8 mAb 321-2, and analyzed by flow cytometry. The graph shows percentages of IL-8⁺ cells out of total PBMC. Individual horse values and medians are graphed for allergic (closed circles; n = 7) and nonallergic (open circles; n = 7) horses.

FIGURE 6. Basophils and IgE-binding monocytes produce IL-8 after IgE cross-linking. To phenotype IL-8⁺ cells, PBMC were stimulated with IgE mAb 134 for 2 h, fixed, and analyzed by intracellular staining and flow cytometry for expression of IL-8, IgE, MHCII, and CD14. PBMC were first gated by FSC and SSC on doublet-excluded cells. (A) Basophils and IgE-binding monocytes are both IgE⁺ and can be distinguished by MHCII expression patterns. (B) CD14 expression on IgE⁺/MHCII^{low} basophils (top, gray) and IgE⁺/MHCII^{high} monocytes (bottom, black). (C) IL-8 production after anti-IgE cross-linking was analyzed in IgE⁺/MHCII^{low} basophils and (D) IgE⁺/MHCII^{high} monocytes. (E) The percentage of IL-8⁺/IgE⁺/MHCII^{low} basophils out of total PBMC. (F) The percentage of IL-8⁺/IgE⁺/MHCII^{high} IgE-binding monocytes out of total PBMC. (G) The percentage of total basophils and IgE⁺ monocytes out of PBMC after 2-h stimulation with IgE mAb 134. Individual horse values and medians are graphed for allergic (closed circles; *n* = 7) and nonallergic (open circles; *n* = 7) horses. Cells cultured in medium alone were included as a control. FACS images all show PBMC-gated cells after 2-h incubation with IgE mAb 134.



the environment and allergic horses looked clinically healthy. Therefore, the differences in IL-8 production by IgE-binding monocytes between allergic and nonallergic horses were not due to allergen exposure or an existing acute inflammation *in vivo*. The increased potential of IgE-binding monocytes to produce IL-8, rather, suggest a basal difference in the innate immune response of allergic horses. Whether allergic individuals have an intrinsic difference in their IgE-binding monocytes to produce IL-8 and aid the initial development of allergy, or repeated allergic episodes enhance the inflammatory and reactive ability of IgE-binding monocytes remains to be determined. In addition, seasonal variations are likely contribute to IL-8 production by equine PBMC. It remains to be evaluated in future studies whether allergic horses produce elevated amounts of IL-8 during *Culicoides* allergen exposure, when they have clinical signs of allergy and are actively experiencing chronic skin inflammation and repair. The horse will be an efficient model to answer these questions because of the ability to collect repeated blood samples from the same horse over long periods of time.

In conclusion, equine IL-8 is produced following cross-linking of FcεRI-bound IgE in IgE-binding monocytes and in basophils. This provides a link between allergen-specific IgE and its ability to upregulate inflammation through innate immune cells to maintain and/or increase allergic symptoms after allergen exposure. IL-8 is well established as a chemokine promoting recruitment of immune cells to the site of inflammation, and the data presented in this study support that IgE-dependent mechanisms can induce IL-8. Horses with an IgE-mediated allergic disease, *Culicoides* hypersensitivity, had

higher percentages of IL-8⁺ IgE-binding monocytes upon IgE cross-linking compared with healthy controls. Most CD14⁺/MHCII⁺/CD16⁺ blood monocytes also produced IL-8 after stimulation with LPS or mitogens, and LPS stimulation also induced higher percentages of IL-8⁺ total monocytes in allergic individuals. These data suggest differences in blood monocyte heterogeneity and/or function in allergic and nonallergic horses and highlight a novel mechanism for IgE-binding monocytes in promoting allergic inflammation through IL-8 production.

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Disclosures

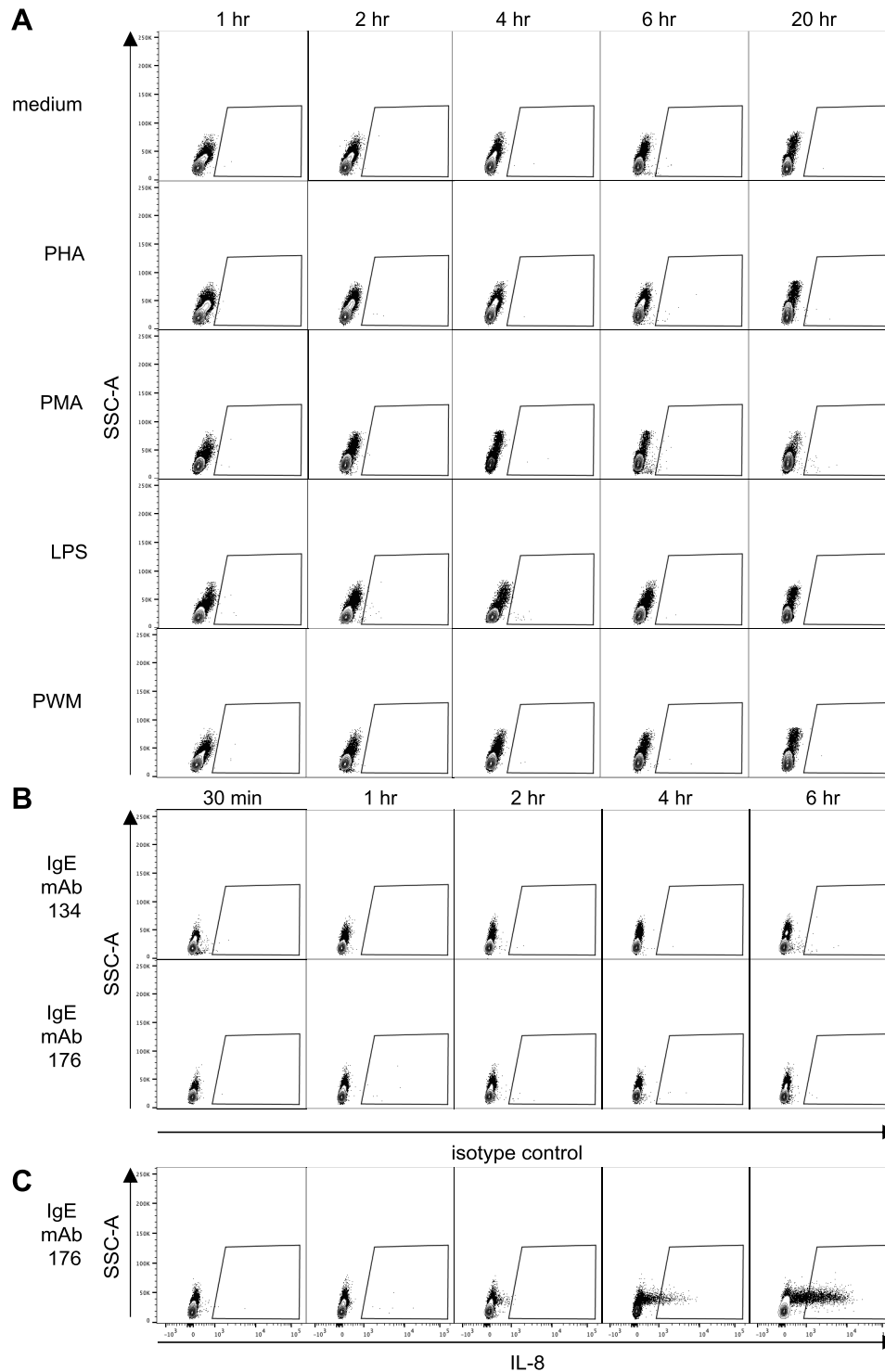
The authors have no financial conflicts of interest.

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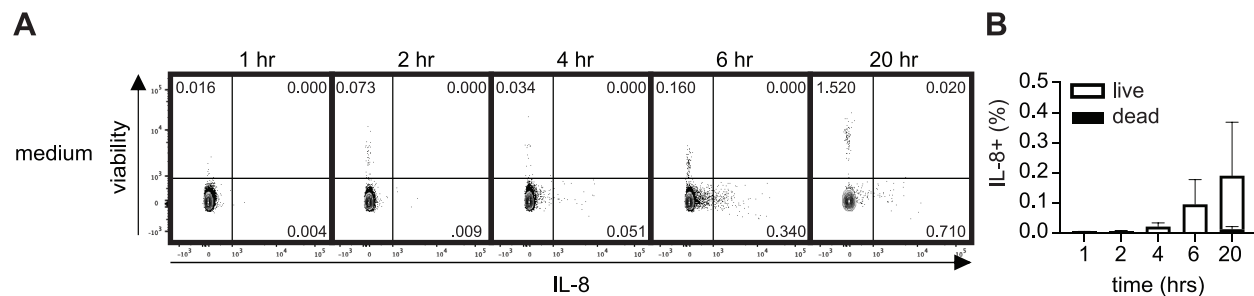
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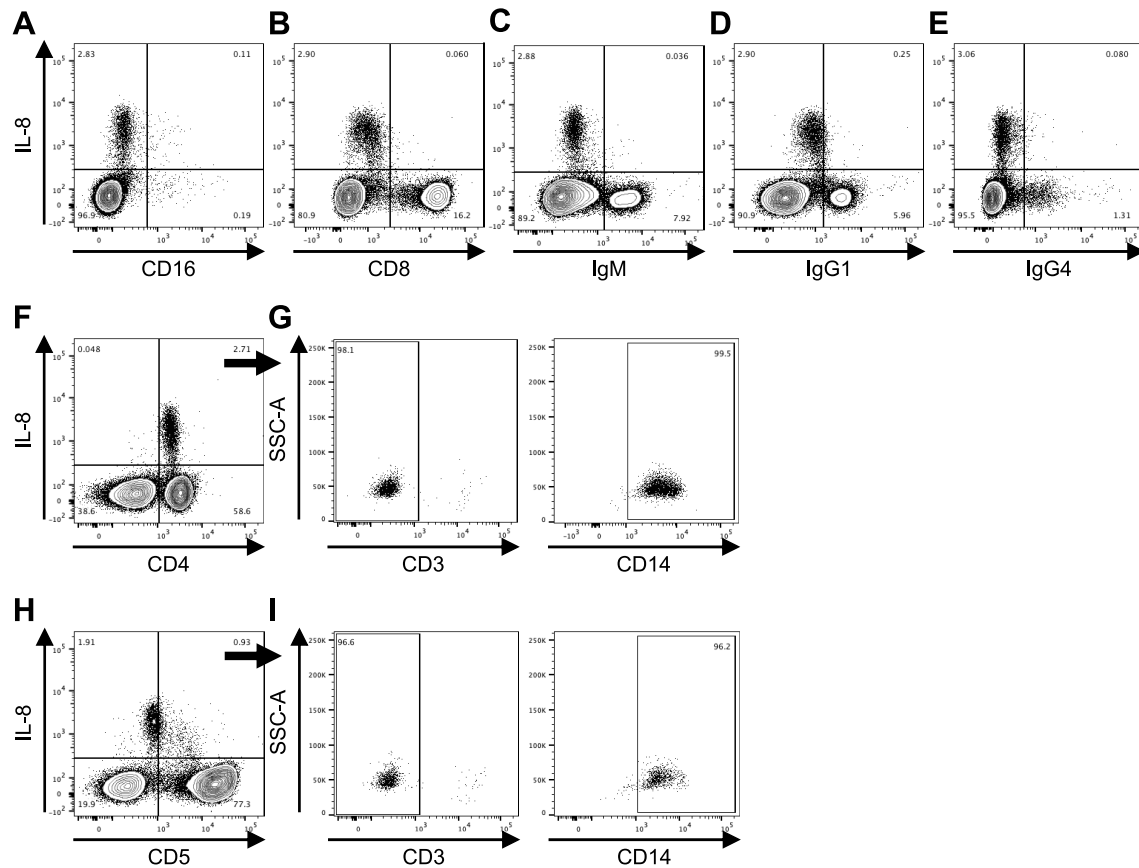
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Supplemental Figure 1: Isotype control does not label native IL-8 in equine PBMC. Stimulated PBMC were fixed, stained intracellularly with an IgG isotype control and analyzed by flow cytometry. (A) PBMC were kept in medium alone or were stimulated with PHA, PMA and ionomycin, LPS or PWM for 1, 2, 4, 6 hours, and overnight (~20 hours). (B) PBMC were stimulated with IgE mAb 134 or IgE mAb 176 for 30 min, 1, 2, 4, and 6 hours. (C) PBMC stimulated with IgE mAb 176 for 30 min, 1, 2, 4, and 6 hours, and were fixed and stained intracellularly with IL-8 mAb 321-2. FACS images show cells in the PBMC gate and are representative images from one out of four horses.



Supplemental Figure 2: Spontaneous IL-8 production after culture in medium occurs in live cells. PBMC were cultured in medium alone for 1, 2, 4, 6 hours, and overnight (~20 hours). After culture, live cells were labeled with a live/dead viability stain, then fixed and stained intracellularly with IL-8 mAb 321-2. Cells negative for viability stain are considered alive. (A) Viability and IL-8 expression for cells in the PBMC gate at each timepoint. (B) The percentage of IL-8+ cells out of total PBMC is summarized for 4 horses. Bars are split to show live (open bars) and dead (closed bars) cells, and show means and SEM.



Supplemental Figure 3: Peripheral B cells, T cells and CD16+ cells do not produce IL-8.

PBMC were stimulated for 2 hours with LPS and stained intracellularly with IL-8 mAb 321-2 and with mAbs for different cell surface proteins: (A) CD16 and IL-8; (B) CD8 and IL-8; (C) IgM and IL-8; (D) IgG1 and IL-8; (E) IgG4 and IL-8; (F) CD4 and IL-8; (G) CD3 and CD14 expression on CD4+/IL-8+ cells; (H) CD5 and IL-8; (I) CD3 and CD14 expression on CD5+/IL-8+ cells. FACS images show PBMC gated cells unless specified otherwise and are representative images from one out of four horses.