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Journal

British Journal of Pharmacology, 179(2)

ISSN

0007-1188

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Publication Date

2022

DOI

10.1111/bph.15721

Peer reviewed

HHS Public Access

Author manuscript

Br J Pharmacol. Author manuscript; available in PMC 2022 January 27.

Published in final edited form as:

Br J Pharmacol. 2022 January ; 179(2): 322–336. doi:10.1111/bph.15721.

Targeting the OXE receptor with a selective antagonist inhibits allergen-induced pulmonary inflammation in non-human primates

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Abstract

Background and Purpose: The 5-lipoxygenase product, 5-oxo-ETE (5-oxo-6,-8,11,14-eicosatetraenoic acid), is a potent chemoattractant for eosinophils and neutrophils. However, little is known about its pathophysiological role because of the lack of a rodent ortholog of the oxoeicosanoid (OXE) receptor. The present study aimed to determine whether the selective OXE receptor antagonist ***S*-Y048** can inhibit allergen-induced pulmonary inflammation in a monkey model of asthma.

Experimental Approach: Monkeys sensitized to house dust mite antigen (HDM) were treated with either vehicle or ***S*-Y048** prior to challenge with aerosolized HDM, and bronchoalveolar (BAL) fluid was collected 24 h later. After 6 weeks, animals that had initially been treated with vehicle received ***S*-Y048** and vice versa for animals initially treated with ***S*-Y048**. Eosinophils and neutrophils in BAL and lung tissue samples were evaluated, as well as mucus-containing cells in bronchi.

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AUTHOR CONTRIBUTIONS

C.C. helped to coordinate the experiments, measured plasma concentrations of antagonist, and evaluated tissue sections using histochemistry and immunohistochemistry. L.A.M. coordinated the in vivo experiments and evaluated BAL fluid cells. Q.Y., S.C., and C.N.R. performed the chemical syntheses. W.S.P., L.A.M., and J.R. designed the study. The manuscript was written by W.S.P with contributions from C.C., L.A.M., and J.R..

CONFLICT OF INTEREST

W.S.P. and J.R. have applied for a patent covering ***S*-Y048**.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

Key Results: HDM significantly increased the numbers of eosinophils, neutrophils, and macrophages in BAL fluid 24 h after challenge. These responses were all significantly inhibited by ***S*-Y048**, which also reduced the numbers of eosinophils and neutrophils in lung tissue 24 h after challenge with HDM. ***S*-Y048** also significantly reduced the numbers of bronchial epithelial cells staining for mucin and MUC5AC after antigen challenge.

Conclusion and Implications: This study provides the first evidence that 5-oxo-ETE may play an important role in inducing allergen-induced pulmonary inflammation and could also be involved in regulating MUC5AC in goblet cells. OXE receptor antagonists such as ***S*-Y048** may useful therapeutic agents in asthma and other eosinophilic as well as neutrophilic diseases.

Keywords

5-lipoxygenase products; 5-oxo-ETE; asthma; eicosanoids; eosinophils; lungs; OXE receptor antagonists

1 | INTRODUCTION

Evidence for the involvement of products of the 5-lipoxygenase (5-LO) pathway in asthma goes back to the 1930s, with the discovery of the release of slow-reacting substance of anaphylaxis (SRS-A) from perfused lungs from sensitized guinea pigs in response to allergen challenge (Kellaway & Trethewie, 1940). SRS-A was subsequently identified as leukotrienes (LT) D4 (Samuelsson et al., 1980), the formation of which was initiated by the oxidation of arachidonic acid to the intermediate LTA4 by 5-LO in the presence of 5-lipoxygenase activating protein (Figure 1). LTD4 is a potent bronchoconstrictor and proinflammatory mediator that acts through the cysLT1 receptor (Lynch et al., 1999), which is the target of selective cysLT$_1$ antagonists such as montelukast, which are widely used in the treatment of asthma (Powell, 2021).

Although the eosinophil plays an important pathological role in many asthmatics, cysLTs have only very modest chemoattractant effects on these cells (Powell et al., 1995). Similarly, the potent neutrophil chemoattractant LTB4 is only a very weak chemoattractant for human eosinophils (Powell et al., 1995; Schwenk et al., 1992; Sun et al., 1991), despite its potent effects on guinea pig eosinophils (Sun et al., 1991). In contrast, another 5-LO product, 5-oxo-ETE (5-oxo-6,-8,11,14-eicosatetraenoic acid), is a potent chemoattractant for human eosinophils, both in vitro (Powell et al., 1995) and in vivo, following subcutaneous injection (Muro et al., 2003). Although the eosinophil is a major target of 5-oxo-ETE, it also has chemoattractant effects on other inflammatory cells, including neutrophils (Powell et al., 1993), basophils (Iikura et al., 2005; Sturm et al., 2005), and monocytes (Sozzani et al., 1996). These actions are mediated by the highly selective OXE receptor, which is encoded by the *OXER1* gene (Bäck et al., 2014; Hosoi et al., 2002; Jones et al., 2003; Takeda et al., 2002). 5-Oxo-ETE is formed following reduction of the LTA$_4$ precursor 5S-HpETE (5*S*-hydroperoxy-6,8,11,14-eicosatetraenoic acid) to 5S-HETE (5*S*-hydroxy-6,8,11,14-eicosatetraenoic acid), which is then oxidized by 5-hydroxyeicosanoid dehydrogenase (5-HEDH) in the presence of the obligatory cofactor NADP$^+$ (Powell et al., 1992). The synthesis of 5-oxo-ETE is thus tightly regulated, as it requires both the activation of 5-LO and elevation of intracellular NADP$^+$, which is normally present only at very low

concentrations, in contrast to its reduced form, NADPH. Conditions present at inflammatory loci, including oxidative stress, cell death, and, in the case of phagocytic cells, the oxidative burst, all promote 5-oxo-ETE formation by elevating the intracellular levels of $NADP^+$ (Powell & Rokach, 2020).

Because of its potent effects on eosinophils, we postulated that 5-oxo-ETE may play a major role in asthma and other allergic diseases that are associated with eosinophil infiltration. Further support for a role of 5-oxo-ETE in asthma comes from a recent study by Kowal et al. showing that allergen challenge of asthmatic subjects who were sensitive to house dust mite allergen (HDM) resulted in increased levels of 5-oxo-ETE in exhaled breath condensate (Kowal et al., 2017). Furthermore, there was a positive correlation between the levels of 5-oxo-ETE and the magnitude of the subsequent late asthmatic response. Progress in the understanding of the pathophysiological role of 5-oxo-ETE has been severely hampered by the lack of an ortholog of the OXE receptor in rodents. To circumvent this problem, we initiated a program to identify small-molecule OXE receptor antagonists that could block the proinflammatory effects of 5-oxo-ETE and potentially serve as novel therapeutic agents in the treatment of asthma and other eosinophilic diseases. To accomplish this, we prepared a series of compounds in which the essential first five carbons of 5-oxo-ETE, along with an alkyl or arylalkyl group mimicking the ω-end of the molecule, were placed on an indole scaffold. The most potent of these compounds is the *S*-enantiomer of **Y048** (i.e., S-Y048, Figure 1), which has an IC_{50} of 20 pM in blocking 5-oxo-ETE-induced calcium mobilization in human neutrophils (Ye et al., 2020). ***S*-Y048** has a long lifetime in the circulation and is slowly converted to an α*S*-hydroxy metabolite (***S*-Y048M**) with equivalent potency.

Because it was not possible to examine the efficacy of OXE receptor antagonists in mouse or rat models, we needed to search for other suitable animal models. We initially considered cats because they are highly susceptible to asthma (Dye et al., 1996). We found that although 5-oxo-ETE is a highly potent activator of feline eosinophils and neutrophils, our OXE receptor antagonists were only relatively weak inhibitors of these responses (Cossette et al., 2015), presumably due to differences between the feline and human receptors, which are about 75% identical. We then explored the monkey, which has an OXE receptor ortholog that has about 95% sequence identity with the human receptor and responds almost identically to 5-oxo-ETE and OXE receptor antagonists (Ye et al., 2020). Although the high cost of these animals severely restricts the scope of experiments that can be performed, the use of primates has the important advantage that the results should be much more transferable to humans (Coffman & Hessel, 2005). In a proof-of-principle study utilizing six rhesus monkeys, we recently showed that ***S*-Y048** inhibits HDM-induced skin eosinophilia (Miller et al., 2020). In the present study, we used these animals, along with six additional monkeys, to determine whether ***S*-Y048** can inhibit pulmonary inflammation following aerosol challenge with HDM.

2 | METHODS

2.1 | Compliance with requirements for studies using animals

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of*

Pharmacology (Lilley et al., 2020). In vivo studies were conducted at the California National Primate Research Center, University of California, Davis. Care and housing of animals before, during, and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and conformed to practices established by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The study was approved by the UC Davis Institutional Animal Care and Use Committee.

2.2 | Animals

Twelve colony-bred adult 7–8 years old male rhesus monkeys were housed indoors in cages with a 12 h light/12 h dark schedule. Indoor cages were of stainless-steel construction and either wall or rolling-rack mounted. Cage sizes were based upon the weight of the animals in accordance with the policies of the United States Department of Agriculture and the National Institutes of Health. Cage designs incorporated sliding partitions to allow socialization or pair housing. All animals in the study were pair-housed whenever possible based upon social compatibility. They received a diet consisting of Purina monkey chow supplemented with produce and had continuous access to water.

The monkeys used for these experiments included the six animals (11.3–6.7 kg) that had been used in our recent proof-of-principle study in which we demonstrated that ***S*-Y048** inhibits HDM-induced skin eosinophilia (Miller et al., 2020). From our experience with the skin study, we anticipated that there would be considerable variability among animals, especially in view of the more complex nature of the present study, so we strengthened it by the inclusion of six additional naïve monkeys (7.5–14.7 kg) that had not previously been exposed to HDM.

2.3 | Sensitization of monkeys to HDM

The monkeys were sensitized by a series of subcutaneous injections of HDM followed by exposure to a combination of subcutaneous and aerosolized HDM. The naïve monkeys were first treated with seven biweekly subcutaneous injections of HDM (*Dermatophagoides pteronyssinus*, Greer Labs; 60-μg protein with 1-mg alum [Imject Alum Adjuvant, Thermo Fisher Scientific; 1-ml total volume per injection]) (Weeks 0 to 12 in Figure 2a). Exposure of these animals to aerosolized HDM was initiated 14 weeks after the first subcutaneous injection of HDM.

The animals that had been used for the prior skin study did not undergo the above regime of treatment with subcutaneous HDM because they had already been sensitized by four biweekly subcutaneous injections of HDM as described previously, followed by challenge by intradermal injection of HDM on three occasions, 4, 9, and 14 weeks after the last of the four subcutaneous injections (Miller et al., 2020). As part of the skin study, they had been treated with two doses of vehicle, administered 0.5 h before and 8 h after HDM (Week 4), followed by 2×5 mg·kg^{-1} ***S*-Y048** (Week 9) and 2×10 mg·kg^{-1} ***S*-Y048** (Week 14). Twenty-four hours after HDM challenge, skin punch biopsies were taken for evaluation of skin eosinophilia (see Miller et al., 2020, for further details). Eighteen days after the last skin biopsy, these six monkeys were entered into the present study and were exposed to

aerosolized HDM as described below (equivalent to Week 14 in the treatment schedule for the naïve monkeys in Figure 2a). After commencement of exposure of the monkeys to aerosolized HDM, the naïve and skin study groups were treated identically and received a total of eight biweekly exposures to aerosolized HDM as shown in Figure 2a, followed 24 h later by subcutaneous injection of HDM.

2.4 | Exposure of monkeys to aerosolized HDM

HDM was diluted in PBS, with a final dose of 1.5-mg total protein per exposure, which was administered using a nebulizer. Animals were sedated with ketamine (5 to 30 $mg \cdot kg^{-1}$) and dexmedetomidine (7.5 to 15 $\mu g \cdot kg^{-1}$) and placed in a child safety seat in a semi-upright position and fitted with a mask that covered both the nose and mouth. A mouth block was placed to ensure maximal aerosol passage into the trachea and lungs. Mask fit and head position were carefully adjusted to prevent leakage of aerosol without occluding the airway. HDM aerosol was administered through the face mask for approximately 5–15 min. Heart rate and oxygen saturation were continuously monitored throughout the procedure. Following the procedure, sedation was reversed by intramuscular injection of atipamezole (dose volume equivalent to that of dexmedetomidine).

2.5 | Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed on four occasions for each monkey: 24 h before and 24 h after the fifth and eight exposures to aerosolized HDM (Figure 2b,c). Monkeys were anaesthetised with ketamine (10 $mg \cdot kg^{-1}$), and anaesthesia was maintained with propofol (0.1 $mg \cdot kg^{-1} \cdot min^{-1}$). Endotoxin-free PBS (10 ml; Sigma, St. Louis, MO) was instilled through a bronchoscope as described previously (Schelegle et al., 2001). A blood sample (2 ml) was obtained immediately prior to each lavage procedure. Lavage samples were cytocentrifuged, air dried, and stained with a modified Wright's stain, and the numbers of macrophages, neutrophils, eosinophils, lymphocytes, and monocytes were determined by counting approximately 300 cells per sample by light microscopy. A single technician performed all lavage cell differentials in a blinded manner on each sample twice, and averages of the two differentials were used for analysis. Prior to evaluation, slides were coded so that the observer was unaware of the identity of the original animal and the treatment group. Macrophages were the predominant population in lavage fluid and identified based upon their large size, heterochromatic oval-shaped and indented nuclei, and pink cytoplasm containing numerous lysosomes. Monocytes were distinguished from macrophages based upon the identification of a horseshoe-shaped nucleus typically not centred in the cell and a muddy grey cytoplasm containing fine granules.

2.6 | Preparation and administration of *S*-Y048

Monkeys were treated with two doses, 8 h apart, of either vehicle or ***S*-Y048** (10 $mg \cdot kg^{-1}$), administered 1 h before and 7 h after challenge with aerosolized HDM on Weeks 22 and 28 (Figure 2a). ***S*-Y048** was synthesized as described previously (Ye et al., 2020), dissolved in ethanol (50 $mg \cdot kg^{-1}$) and stored at −80°C prior to use. On the morning of the experiment, the ethanolic solution was thawed and vortexed to dissolve any ***S*-Y048** that had precipitated out, and the required amount was added to 10 volumes of 20-mM $NaHCO_3$ (pH 8.0). The

resulting suspension (2.2 ml·kg^{-1}; 9.1% EtOH) was immediately vortexed and administered by nasogastric intubation 1 h before and 7 h after challenge with aerosolized HDM (Figure 2b,c). For nasogastric intubation prior to HDM, animals were sedated as described for mask aerosol exposures (see prior section). For nasogastric intubation at 7 h after HDM challenge, animals were sedated with ketamine at 10 mg·kg^{-1}. Control animals were treated identically except that they received the bicarbonate/ethanol vehicle without ***S*-Y048**. Blood samples (2 ml) were taken immediately before HDM exposure, immediately before the second dose of ***S*-Y048**, and 24 h after HDM challenge for measurement of plasma ***S*-Y048** concentrations by reversed-phase HPLC as described previously (Ye et al., 2020).

2.7 | Study design

BAL fluid was obtained 24 h before challenge with aerosolized HDM on Week 22 (Figure 2a,b). Either vehicle or ***S*-Y048** (10 mg·kg^{-1}) was administered 1 h prior to and 7 h after exposure to aerosolized HDM, and a second sample of BAL fluid was obtained 24 h after exposure to HDM. This procedure was repeated 6 weeks later (Week 28, Figure 2a,c) except that after the second BAL fluid sample was obtained, the animals were deeply anaesthetised with an overdose of intravenous sodium pentobarbital (greater than or equal to 120 mg·kg^{-1}) and euthanized by exsanguination via the systemic aorta. Samples of lung (right middle lobe) and bronchus were collected at necropsy, fixed in paraformaldehyde for 48 h, and embedded in paraffin.

To minimize the effects of variability among monkeys, a crossover design was used, permitting each monkey to serve as its own control (Figure 2d). In addition to biological variability among individual monkeys, factors that could theoretically affect the outcome of the experiment could be whether or not they had previously been used in the prior skin study and whether they received vehicle first or ***S*-Y048** first. To minimize any bias due to these factors, on Week 22, three naïve animals and three of the animals from the skin study were treated with vehicle first and the remaining three animals from each group were treated with ***S*-Y048** first (Figure 2d). Six weeks later, on Week 28, the animals that had received vehicle on Week 22 were treated with ***S*-Y048** whereas the animals that had received ***S*-Y048** on Week 22 were treated with vehicle. The animals were assigned to receive either vehicle first or ***S*-Y048** first according to their original cage number, with the lower cage numbers receiving vehicle first. However, there were no further randomization procedures. The cage numbers to which the animals had originally been assigned were not related to any physical characteristics of the monkeys. There were no significant differences in weight or skin sensitivity to HDM just prior to aerosol exposure between the animals that received vehicle first and those that received ***S*-Y048** first.

2.8 | Evaluation of eosinophils and neutrophils in lung tissue sections

Eosinophils and neutrophils were evaluated in 5-μm-thick sections (~2 cm^2) prepared from paraffin blocks. Prior to staining, the sections were deparaffinized in xylene and dehydrated in ethanol. Eosinophils were stained with haematoxylin and eosin, whereas neutrophils were detected using naphthol AS-D chloroacetate, which is hydrolysed by neutrophil-specific esterase, resulting in a red stain after treatment with new fuchsin (Schön et al., 2000). Briefly, 4% $NaNO_2$ (300 μl; Sigma-Aldrich, St. Louis MO) was mixed with New Fuchsin

in 2-N HCl (300 μl; Sigma-Aldrich). An aliquot (500 μl) of this solution was then added to a mixture of naphthol AS-D chloroacetate (10 mg in 5-ml dimethylformamide [Sigma-Aldrich]) and PBS (100 ml). The slides were immersed in this solution for 45 min at 23 °C and counter-stained with haematoxylin and lithium carbonate. Sections were viewed using a Zeiss microscope (400x magnification). All positive cells around the airways were counted by a research technician in a blinded manner for each of the sections using Image J software. Each section was coded prior to analysis so that the observer was unaware of the identity of the original animal and the treatment group. The results are expressed as the numbers of positive cells per millimetre basement membrane.

2.9 | Histochemical evaluation of mucus-containing cells in bronchi

Sections of bronchi were deparaffinized as described above and stained with Alcian Blue (AB), followed by periodic acid and Schiff reagent (PAS) according to the manufacturer's instructions using a kit from ScyTek Laboratories, West Logan, UT. Sections were viewed using a Zeiss microscope (200× magnification). All positive epithelial cells within the entire section were counted by a technician in a blinded manner as described above using Image J software. The results are expressed as the numbers of positive cells per millimetre basement membrane.

2.10 | Evaluation of MUC5AC positive cells by immunohistochemistry

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018). To visualize MUC5AC, sections were deparaffinized in xylene, dehydrated in ethanol, and washed with Tris-buffered saline (TBS). Antigen retrieval was facilitated by incubation for 40 min at 97°C with Tris-EDTA (pH 9) containing 0.05% Tween 20. After washing with PBS, the sections were incubated with 5% rabbit normal serum in PBS for 30 min, followed by incubation overnight at 4°C with a monoclonal antibody against MUC5AC (clone 45M1; Novus Biologicals, Centennial, CO; catalogue number NBP2-15196, diluted 1:500). The slides were then washed with PBS (3 × 5 min) and incubated with the secondary antibody (biotinylated polyclonal rabbit anti-mouse IgG (Dako E0354, lot 00078773, diluted 1:100) for 1 h at 23°C. After washing with PBS (3 × 5 min), the slides were incubated for 30 min at 23°C with Roche AP-Streptavidin diluted 1:400 (Sigma-Aldrich), washed with PBS (3 × 5 min), and visualized by incubation for 15 min at 23°C with ImmPACT Vector Red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA). After counterstaining with haematoxylin and lithium carbonate, the sections were dehydrated by treatment with ethanol followed by xylene. All positive epithelial cells within the entire section were counted by a technician in a blinded manner as described above using Image J software and the results expressed as the numbers of positive cells per millimetre basement membrane.

2.11 | Statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis as described in the literature (Curtis et al., 2018). The BAL cell data shown in Figure 4 were not normally distributed and were analysed by Friedman repeated measures analysis of variance on ranks with the Student-Newman–Keuls method as a post hoc test using SigmaStat software. The Grubbs'

outlier test (Grubbs, 1969) was used to detect outliers using GraphPad software. Comparison of two groups (Figures 5–7) was evaluated with SigmaStat software using Student's *t* test for normally distributed data or the Wilcoxon signed rank test for data that were not normally distributed. The precise statistical test used is described in each of the relevant figure legends. The threshold for statistically significant differences was $P < 0.05$.

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

The primary goal of this study was to determine whether ***S*-Y048** can inhibit allergen-induced pulmonary inflammation, by comparing the numbers of inflammatory cells in BAL fluid following treatment of animals with either vehicle or ***S*-Y048** prior to allergen challenge. To accomplish this, we used six rhesus monkeys that we had previously employed in a pilot study to demonstrate the efficacy of ***S*-Y048** in inhibiting allergen-induced skin eosinophilia (Miller et al., 2020). To strengthen the study, we included six additional naïve monkeys that were initially sensitized by subcutaneous injection of HDM as had been done with the monkeys used in the skin study. All 12 monkeys were then further sensitized by four biweekly exposures to aerosolized HDM (Figure 2a). On Week 22, monkeys were treated with either vehicle or ***S*-Y048** (2×10 mg·kg^{-1}) and then challenged with aerosolized HDM as illustrated in Figure 2b,d and described in more detail in Section 2. The numbers of inflammatory cells in BAL fluid collected 24 h before and 24 h after antigen challenge were compared. This procedure was repeated on Week 28, except that the animals that had first received vehicle were treated with ***S*-Y048** (Figure 2c). After collection of the final BAL fluid samples, the animals were euthanized, and samples of lung and bronchi were obtained at necropsy.

3.1 | *S*-Y048 levels and inflammatory cell numbers in blood

To ensure that adequate blood levels of antagonist were obtained during these experiments, we measured the plasma levels of ***S*-Y048** and its major metabolite by reversed-phase HPLC. The average concentration of ***S*-Y048** in plasma rose to about 16 μM 1 h after administration, diminished slightly to about 14 μM just prior to administration of the second dose of ***S*-Y048**, and was about 28 μM after 24 h (Figure 3a). The levels of ***S*-Y048M**, the main plasma metabolite of ***S*-Y048**, were initially very low, but rose to nearly 5 μM by 24 h.

The numbers of eosinophils (Figure 3b) and neutrophils (Figure 3c) were measured in blood taken 24 h before and 24 h after HDM challenge of vehicle- and ***S*-Y048**-treated monkeys, but no significant differences were observed. Alterations in blood granulocytes numbers could have occurred at earlier time points, but this study was not designed to investigate this possibility.

3.2 | *S*-Y048 reduces the numbers of inflammatory cells in BAL fluid following allergen challenge

Challenge of vehicle-treated sensitized monkeys with HDM significantly increased the numbers of BAL cells (Figure 4a), including macrophages (Figure 4b), neutrophils (Figure 4c), and eosinophils (Figure 4f), but not monocytes (Figure 4d) nor lymphocytes (Figure 4e). Total BAL cell numbers increased by over threefold in response to HDM in vehicle-treated animals, but remained unchanged in ***S*-Y048**-treated monkeys (Figure 4a). Similarly, HDM elicited an almost threefold increase in the numbers of macrophages, the major cell type in BAL fluid, in the group that received vehicle but had no effect on macrophage numbers in the ***S*-Y048**-treated group (Figure 4b). Neutrophils underwent a dramatic 24-fold increase in the vehicle-treated group as a result of HDM challenge, but only a sixfold increase in the group that received ***S*-Y048**, amounting to 77% inhibition ($P < 0.05$; Figure 4c).

Eosinophils in BAL fluid increased significantly by about 4.5-fold in response to HDM in vehicle-treated animals, and by about 3-fold in animals treated with ***S*-Y048**, equivalent to 52% inhibition ($P < 0.05$; Figure 4f). The BAL eosinophil data for each individual animal are shown in Figure 4g,h. Because eosinophil numbers varied widely from one animal to another, data from three individual animals with total BAL eosinophils above 10^7 for at least one of the four time points examined are shown separately in Figure 4g, whereas data from the remaining nine animals, in which eosinophil numbers never exceeded 0.6×10^7, are shown in Figure 4h. The BAL eosinophil data for one of the 12 monkeys, shown with "X" symbols in Figure 4g, does not make sense. This was a naïve monkey that had been treated with vehicle first on Week 22 and with ***S*-Y048** on Week 28. According to the data for Week 22, baseline pre-challenge eosinophil numbers for this monkey were extremely high (total $>10^7$ cells), which was over five times higher than any of the other monkeys in the study. However, what was even more unusual was that exposure of this monkey to HDM appeared to *reduce* the number of BAL eosinophils by 60%. This is counterintuitive, as the model of pulmonary inflammation used in the present study is based on the presumption that an animal sensitized to HDM will respond to HDM challenge with increased, rather than decreased, inflammation. We do not know the reason for this discrepancy. It is possible that there was a mistake in labelling or alternatively, there may have been some basic difference with this particular monkey. The post-challenge eosinophil numbers after treatment with ***S*-Y048** were also very high, over twice that of any of the other monkeys. Because of the aberrant eosinophil data from this monkey, it could be regarded as an outlier. Analysis of the eosinophil data using Grubbs' outlier test revealed that for this monkey, two of the eosinophil data points ("before HDM with vehicle" and "after HDM with ***S*-Y048**", labelled with arrows and "***a***") were outliers ($P < 0.01$; Figure 4g). One additional outlier was detected using Grubbs' test ($P < 0.05$; "after vehicle and HDM") and is indicated with an arrow and the letter "***b***." Figure 4i shows BAL eosinophil numbers after removal of the data from the monkey with the outliers labelled "***a***" (i.e., $n = 11$). In this case, HDM significantly increased BAL eosinophils by over 12-fold in vehicle-treated animals compared with just over twofold after treatment with ***S*-Y048** (80% inhibition; $P < 0.05$). Even if all of the data from the two monkeys with outlying data points are removed (i.e., $n = 10$), ***S*-Y048** still significantly reduced the numbers of eosinophils in BAL fluid by 69% (Figure 4j). It should

be noted that, with the exceptions of Figure 4i,j, data from all 12 monkeys are shown in all of the graphs in Figures 3–7.

Data for the numbers of eosinophils, neutrophils, and macrophages in individual animals showing each of the four different subgroups illustrated in Figure 2d are shown in Figure 5. To simplify the data and facilitate comparison of animals from different subgroups, “delta” values (i.e., number of cells after HDM minus number of cells before HDM) are shown, representing HDM-induced increases in cell numbers. A positive number would mean that exposure to HDM increased BAL cell numbers, whereas a negative number would mean that cell numbers were lower after HDM.

S*-Y048** reduced the eosinophil response to HDM in five out the six animals in the skin study group and in four of the six naïve animals (Figure 5a). Among those animals that received vehicle first (open symbols), ***S*-Y048** reduced HDM-induced BAL eosinophilia in three out of the six animals compared with six out of six of the animals that were treated with ***S*-Y048** first. Overall, BAL eosinophil numbers increased by an average of $47 \pm 24 \times 10^5$ cells after challenge with HDM in the presence of vehicle and $23 \pm 13 \times 10^5$ cells in the presence of ***S*-Y048** (not significantly different). However, when outlier ***a is excluded, the increases in BAL eosinophils were 57 ± 24 and $11 \pm 7 \times 10^5$ cells in the presence of vehicle and ***S*-Y048**, respectively ($P < 0.05$).

Similar, but more pronounced results were obtained with neutrophils (Figure 5b). ***S*-Y048** inhibited the effect of HDM on BAL neutrophils in all six of the animals in the skin study group and in three of the naïve animals. ***S*-Y048** reduced the neutrophil response in four of the six animals that received vehicle first and in five of the animals that received ***S*-Y048** first. As in Figure 5a, the outlier referred to above is indicated by inverted open triangles connected by dashed lines in Figure 5b,c. In contrast to the eosinophil data, HDM increased the number of BAL neutrophils in this monkey in the presence of vehicle. However, this animal was one of the three in which the response to HDM was greater in the presence of ***S*-Y048**. The two animals with the greatest eosinophil response to HDM in the presence of vehicle (Figure 5a) also exhibited the greatest neutrophil response (Figure 5b), which, in both cases, was strongly inhibited by ***S*-Y048**. Overall, ***S*-Y048** significantly reduced the neutrophil response to HDM from $92 \pm 33 \times 10^5$ to $21 \pm 10 \times 10^5$ cells.

Among the monkeys that had been used for the skin study, ***S*-Y048** reduced HDM-induced macrophage infiltration in the three animals that had received ***S*-Y048** first, but, in contrast to granulocytes, in none of those that had received vehicle first. The effect of HDM was reduced in five of the six naïve monkeys and in three of the six monkeys from the skins study. Overall, ***S*-Y048** reduced the macrophage response from 180 ± 80 to -10 ± 30 cells (not significantly different).

3.3 | *S*-Y048 inhibits HDM-induced lung tissue eosinophilia and neutrophilia

At the end of the experiment, the animals were euthanized immediately after the final BAL procedure, and samples of lung and bronchi were obtained at necropsy to evaluate tissue inflammation. All of these animals had been challenged with HDM on the previous day. The numbers of eosinophils in lung tissue sections were determined after staining with

haematoxylin and eosin. Large numbers of these cells were detected around the airways in monkeys that had been treated with vehicle prior to inhalation of HDM (Figure 6a), whereas much smaller numbers were detected in monkeys that had been treated with ***S*-Y048** prior to antigen challenge (Figure 6b). The values for individual monkeys are shown in Figure 6c. Overall, ***S*-Y048** reduced lung tissue eosinophils by nearly 50% ($P < 0.05$). The animals from the skin study appeared to respond more strongly than the naïve animals to both HDM and ***S*-Y048**, but the numbers were too small to permit statistical evaluation. Outlier ***a*** (shown in Figure 4g), which was from the naïve group (inverted filled triangle), had the greatest number of eosinophils among those treated with ***S*-Y048**.

The numbers of neutrophils in lung tissue sections obtained as described above were evaluated using naphthol AS-D chloroacetate, which is hydrolysed by neutrophil specific esterase, resulting in red staining of neutrophils following treatment with New Fuchsin. Neutrophils were abundant around the airways of animals that had received vehicle prior to challenge with HDM (Figure 6d), but were present in significantly lower numbers in animals that had been treated with ***S*-Y048** (Figure 6e). As shown in Figure 6f, ***S*-Y048** reduced the numbers of neutrophils in lung tissue by about 50%. The responses to HDM and ***S*-Y048** appeared to be greater in the animals from the skin study compared with the naïve animals, but the numbers were too small for valid statistical comparison.

3.4 | *S*-Y048 reduces the numbers of mucin-positive cells in bronchi from HDM-challenged monkeys

To determine whether ***S*-Y048** affected the numbers of mucus-containing cells in monkey airways following allergen challenge, we stained bronchial sections with AB/PAS. The bronchial epithelium from vehicle-treated monkeys contained a very large number of AB/PAS positive cells (Figure 7a) compared with ***S*-Y048**-treated animals (Figure 7b). Overall, ***S*-Y048** reduced the number of positive cells by about 40% ($P < 0.01$; Figure 7c). We then examined the numbers of bronchial epithelial cells expressing the mucin subtype MUC5AC by immunostaining. We detected considerably greater numbers of MUC5AC-positive cells in bronchial sections from vehicle-treated monkeys (Figure 7d) compared with animals that had received ***S*-Y048** (Figure 7e). Examination of the data from all 12 animals showed that ***S*-Y048** reduced the numbers of bronchial MUC5AC-containing cells by about 60% ($P < 0.05$; Figure 7f).

4 | DISCUSSION

Among lipid mediators, 5-oxo-ETE is the most powerful in vitro chemoattractant for human eosinophils (Powell & Rokach, 2020). It also elicits eosinophil transendothelial migration due to a combination of its chemoattractant effects with stimulation of MMP-9 release (Dallaire et al., 2003; Guilbert et al., 1999). These in vitro data, along with our in vivo experiments (Muro et al., 2003) showing that 5-oxo-ETE induces dermal eosinophilia in humans led us to search for selective OXE receptor antagonists. The identification of S-Y048 as a potent and metabolically resistant OXE receptor antagonist finally gave us the opportunity to directly investigate the pathophysiological role of 5-oxo-ETE, which has in the past been severely hampered by the unavailability of rodent models due to their lack of

an OXE receptor ortholog. ***S*-Y048** is highly selective for the OXE receptor and has no effect on the responses of eosinophils and neutrophils to prostaglandin D_2 (PGD2), LTB4, and eotaxin (Ye et al., 2020). Moreover, the structurally related OXE antagonist, compound **230**, completely blocks 5-oxo-ETE-induced calcium mobilization in neutrophils without affecting the responses to LTB4, platelet-activating factor, fMLP, and interleukin 8 (CXCL8) (Gore et al., 2014).

Because of the lack of an OXE receptor ortholog in rodents and the weak response of the feline OXE receptor to our antagonists, we chose to use the monkey to test their efficacy as anti-inflammatory agents. Initial studies showed that 5-oxo-ETE is a potent in vitro activator of monkey eosinophils and neutrophils, inducing calcium mobilization, actin polymerization, and cell migration. Furthermore, all of these responses are completely blocked in monkey cells by ***S*-Y048** (Miller et al., 2020) and compound **230** (Cossette et al., 2016), indicating that monkey granulocytes express the OXE receptor. Since OXE antagonists had never before been tested in vivo, before initiating our present studies we conducted a pilot experiment, which showed that ***S*-Y048** inhibits HDM-induced skin eosinophilia in rhesus monkeys (Miller et al., 2020). This was the first in vivo evidence in any species other than the zebrafish (Enyedi et al., 2013) for a pathophysiological role for 5-oxo-ETE and the OXE receptor.

The success of the pilot skin study prompted us to investigate the efficacy of ***S*-Y048** in a more complex model, allergen-induced pulmonary inflammation. To accomplish this, we used the six monkeys from the skin study, along with six additional naïve monkeys, which were included because of the anticipated variability among animals. Based on our knowledge of the in vitro and in vivo effects of 5-oxo-ETE on human and monkey eosinophils, our primary goal was to demonstrate that ***S*-Y048** could reduce BAL eosinophil numbers following allergen challenge.

The dose of ***S*-Y048** (2×10 mg·kg^{-1}) used in these experiments was equivalent to the higher dose used in our previous skin study and was based on prior pharmacokinetic studies in both cynomolgus (Ye et al., 2020) and rhesus (Miller et al., 2020) monkeys. In these studies, we found that two doses given 8 h apart resulted in sustained high plasma levels of antagonist over 24 h. Maximal levels were achieved after 1 to 2 h, followed by a nadir just before administration of the second dose. In the present study, HDM was administered 1 h after the first dose of ***S*-Y048** to allow sufficient time to achieve high plasma levels of antagonist prior to antigen challenge. We verified the presence of high levels of ***S*-Y048** in plasma just prior to administration of HDM, just prior to the second dose of ***S*-Y048** to ensure that the levels had not fallen appreciably, and after 24 h to ensure that high levels were maintained throughout the experiment.

The average plasma concentration of ***S*-Y048** varied between about 15 and 30 μM throughout the experiment, well above its IC_{50} value of 20 pM for inhibition of 5-oxo-ETE-induced calcium mobilization in neutrophils. We chose to use a high dose of ***S*-Y048** because it is quite hydrophobic and a considerable portion would likely be bound to protein in the blood, thereby reducing its apparent in vivo potency. Furthermore, ***S*-Y048** is less potent in inhibiting certain other in vitro responses, including actin polymerization (IC_{50},

340 pM) in eosinophils and migration of mixed leukocytes (principally neutrophils; IC_{50}, 30 nM) (Miller et al., 2020). We previously showed in in vitro experiments that ***S*-Y048** at a concentration of 1 μM (which is likely to considerably exceed its free concentration in the blood) has no effect on actin polymerization induced by PGD_2, LTB_4, or eotaxin (Ye et al., 2020). Therefore, we believe that the in vivo effects of ***S*-Y048** that we observed were due to selective blocking of the OXE receptor, although we cannot completely exclude the possibility of off-target effects.

Exposure of monkeys to HDM increased BAL eosinophils in 11 of the 12 monkeys. The remaining monkey appeared to have very high baseline eosinophil numbers, which were reduced, rather than increased, by HDM. It is not clear whether this was due to methodological or biological factors, or even mislabelling. Due to the incongruous eosinophil data from this animal, it can be considered to be an outlier with respect to BAL eosinophils. Nevertheless, ***S*-Y048** significantly reduced BAL eosinophil numbers by an average of about 50% in all 12 monkeys and by about 70% if the outlying data is excluded. To provide additional evidence for the effectiveness of ***S*-Y048** in inhibiting eosinophil infiltration into the lungs, we also evaluated the numbers of these cells in lung tissue sections after allergen challenge. Unlike the BAL data, we only had sections that were obtained post-challenge at Week 28, as it was not possible for cost reasons to include additional control animals that were sensitized but not challenged with HDM prior to sacrifice and necropsy. ***S*-Y048** significantly lowered tissue eosinophil numbers by about 50% compared with vehicle-treated animals. Interestingly, the single ***S*-Y048**-treated monkey with high tissue eosinophil numbers (Figure 6c) was identical to outlier ***a*** referred to above, suggesting that there may have been, at least in part, a biological basis for its aberrant responses. Possibly, in this particular animal 5-oxo-ETE may have contributed less to pulmonary eosinophilia compared with chemokines and cytokines as discussed in more detail below.

HDM appeared to elicit slightly greater numbers of neutrophils in both BAL fluid and lung tissue compared with eosinophils, which may be due to contamination of the HDM extract with small amounts of endotoxin. Although our initial focus was on eosinophils, the presence of substantial numbers of neutrophils, as occurs in some endotypes of asthma (Svenningsen & Nair, 2017), enabled us to demonstrate that ***S*-Y048** also had a strong inhibitory effect on post-challenge neutrophil numbers. Although 5-oxo-ETE is a neutrophil chemoattractant, it is less potent than LTB_4 (Powell et al., 1993), which is usually produced at the same time, so it might have been expected that selectively blocking the response to 5-oxo-ETE would have relatively little effect. However, this was not the case, as ***S*-Y048**, which is inactive against the BLT1 receptor for LTB_4, markedly reduced the numbers of neutrophils in both lung tissue and BAL fluid. This suggests that in this model, there is an increased dependence on 5-oxo-ETE compared with LTB_4 for neutrophil recruitment. This could possibly be due to increased production of 5-oxo-ETE compared with LTB_4 under conditions of oxidative stress (Erlemann et al., 2004; Graham et al., 2009; Grant et al., 2011). It is also possible that the response to 5-oxo-ETE was enhanced compared with LTB_4 following allergen challenge due to synergy with other chemoattractants or cytokines such as GM-CSF (O'Flaherty et al., 1996a).

Although the fold increase in macrophages induced by HDM was much less than that for eosinophils and neutrophils, their numbers were also lowered by ***S*-Y048**. Although there is little information available about the relationship of 5-oxo-ETE to macrophages, it is known to be a chemoattractant for human monocytes and to have synergistic effects with other monocyte chemoattractants (Sozzani et al., 1996). It also stimulates the release of GM-CSF from these cells (Stamatiou et al., 2004), which might in turn enhance OXE-mediated responses to 5-oxo-ETE and increase the survival of neutrophils and eosinophils.

The high degree of variability in the responses to HDM and ***S*-Y048** among the 12 monkeys was not unexpected, since these animals are outbred and would have diverse genetic backgrounds. This could result in differences among individual animals in their abilities to synthesize 5-oxo-ETE and in their expression of the OXE receptor as well as other chemoattractants (e.g., eotaxin, PGD_2, and IL-8), cytokines (e.g., GM-CSF, IL-5, IL-13), and their receptors. GM-CSF, for example, could have multiple effects, as it has important synergistic interactions with 5-oxo-ETE (O'Flaherty et al., 1996b; Stamatiou et al., 2004) and could potentially promote its synthesis (DiPersio et al., 1988; Pouliot et al., 1994). Another factor could be differences in the levels of testosterone, which has been reported to reduce the production of 5-LO products (Pergola et al., 2008) and to selectively inhibit activation of the OXE receptor (Kalyvianaki et al., 2017). The present study utilized male animals, which may not have responded as well as females to ***S*-Y048**. Overall, it is not unusual to have variable responses to drugs in humans, as exemplified by $cysLT_1$ receptor antagonists, which work very well in many human asthmatics, but have little or no effect in others, which may be due to genetic variations among individuals (Lima et al., 2006). To minimize such effects, each animal was used as its own control for the experiments utilizing BAL fluid, but this was not possible for the experiments using lung or bronchial tissue samples.

Another factor that might have contributed to the variability among animals could be the inclusion of the six naïve monkeys. Although these animals were sensitized in a similar manner to those used in the skin study, they were not subjected to intradermal injection of antigen and multiple skin biopsies, as were those in the original pilot study. To permit each animal to serve as its own control they had to be challenged with aerosolized HDM on two separate occasions, 6 weeks apart, and it is possible that the response to HDM differed between these two time points. Based on the delta values shown in Figure 5 and the lung tissue data in Figure 6, the animals that had previously been used for the skin study appeared to respond more strongly to the stimulatory and inhibitory effects of HDM and ***S*-Y048**, respectively. The animals also appeared to respond more strongly to HDM on Week 28 compared with Week 22. However, the small numbers of animals in each of the subgroups ($n = 3$) resulting from the experimental design (Figure 2d) were insufficient to permit us to draw any meaningful statistically-valid conclusions about the precise contributions of the above factors. The crossover design used in this study would have mitigated against any bias due to the above factors, but it would not have reduced their considerable contribution to the variability in the responses among individual animals. Nevertheless, despite this variability, which is somewhat reminiscent of the variability seen in human populations, it can be concluded that ***S*-Y048** inhibits allergen-induced granulocyte infiltration into the lungs.

As increased mucus secretion is a hallmark of asthma (Morcillo & Cortijo, 2006), we stained sections of bronchus from HDM-challenged monkeys with AB/PAS. The reduced staining that we observed in sections from ***S*-Y048**-treated monkeys prompted us to evaluate the numbers of epithelial cells containing MUC5AC because of its association with asthma (Bonser & Erle, 2017). In agreement with previous reports (Okuda et al., 2019), we did not detect appreciable numbers of MUC5AC-positive cells in lung tissue sections but observed considerable numbers in bronchial epithelium, which were significantly reduced in monkeys treated with ***S*-Y048**. These results are very interesting and are the first evidence that 5-oxo-ETE may play a role in regulating the secretion of mucins, either by acting directly on goblet cells or by acting indirectly, possibly by activating eosinophils, which have been shown to increase MUC5AC expression by airway epithelial cells through the release of TGF-α and its interaction with the EGF receptor (Burgel et al., 2001). Although these results suggest that 5-oxo-ETE may increase the production of MUC5AC, as occurs in asthma, they must be interpreted with caution, as we cannot exclude the possibility that the reduction in mucin-positive cells was due to enhanced mucus secretion following treatment with ***S*-Y048**. In this alternative scenario, endogenous 5-oxo-ETE could inhibit the secretion of MUC5AC from goblet cells in response to HDM, and this could be reversed by ***S*-Y048**, resulting greater secretion of MUC5AC and lower numbers of immunostained cells. Clearly, further studies will be required to unravel the potentially important relationship between 5-oxo-ETE, the OXE receptor, and mucus secretion in primates.

In conclusion, the identification of highly potent and selective OXE receptor antagonists has finally made it possible to examine the pathophysiological role of 5-oxo-ETE in mammalian species in vivo. The only other evidence to date implicating 5-oxo-ETE and the OXE receptor in an in vivo disease model is a study by Enyedi et al. showing that 5-oxo-ETE and the OXE receptor play an important role in promoting wound healing by stimulating leukocyte infiltration in a zebrafish model (Enyedi et al., 2013). They found that 5-oxo-ETE enhanced leukocyte infiltration induced by tissue damage, which could be blocked by knockdown of the OXE receptor using a morpholino. The present study, along with our previous skin data, provides the first evidence for an in vivo role of 5-oxo-ETE/OXE in a mammalian species and are the first demonstration of in vivo efficacy of an OXE receptor antagonist in any species. The inhibitory effects of ***S*-Y048** on HDM-elicited eosinophil and neutrophil infiltration and airway mucin production provide the first direct evidence that the OXE receptor may play an important role in allergen-induced pulmonary inflammation. Large numbers of eosinophils and/or neutrophils are characteristic of most endotypes of asthma (Svenningsen & Nair, 2017) and can cause damage due to the release of various enzymes and proinflammatory mediators (McBrien & Menzies-Gow, 2017; Monteseirin, 2009), whereas excessive mucus secretion can contribute to airflow limitation and airway hyperresponsiveness (Morcillo & Cortijo, 2006). Selective OXE receptor antagonists such as ***S*-Y048** that can block the proinflammatory responses to 5-oxo-ETE offer a novel therapeutic approach for the treatment of asthma and other inflammatory diseases.

ACKNOWLEDGEMENTS

This work was supported by the Canadian Institutes of Health Research (W.S.P., Grants MOP-6254 and PP2-133388), the American Asthma Foundation (J.R., Grant 12-0049), and the National Heart, Lung, and Blood

Institute (J.R., Grant R01HL081873) and by AmorChem (Montreal, QC). The Meakins-Christie Laboratories-MUHC-RI are supported in part by a Centre grant from Le Fond de la Recherche en Santé du Québec as well as by the J. T. Costello Memorial Research Fund. J.R. also wishes to acknowledge the National Science Foundation for the AMX-360 (Grant CHE-90-13145) and Bruker 400 MHz (Grant CHE-03-42251) NMR instruments. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

Funding information

National Science Foundation, Grant/Award Numbers: CHE-03-42251, CHE-90-13145; AmorChem; National Heart, Lung, and Blood Institute, Grant/Award Number: R01HL081873; American Asthma Foundation, Grant/Award Number: 12-0049; Canadian Institutes of Health Research, Grant/Award Numbers: PP2-133388, MOP-6254

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abbreviations:

5-oxo-ETE	5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
AB	Alcian Blue
BAL	bronchoalveolar lavage
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDM	house dust mite allergen
PAS	periodic acid and Schiff reagent
***S*-Y048**	(*S*)-5-(5-chloro-2-(6-(3-chlorophenyl)hexyl)-1-methyl-1*H*-indol-3-yl)-3-methyl-5-oxopentanoic acid
***S*-Y048M**	(*S*)-5-(5-chloro-2-((*S*)-6-(3-chlorophenyl)-1-hydroxyhexyl)-1-methyl-1*H*-indol-3-yl)-3-methyl-5-oxopentanoic acid

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What is already known

- The 5-lipoxygenase product 5-oxo-ETE is a potent eosinophil chemoattractant that acts through the OXE receptor.
- The selective OXE receptor antagonist ***S*-Y048** blocks allergen-induced dermal eosinophilia in monkeys.

What does this study add

- ***S*-Y048** inhibits allergen-induced infiltration of both eosinophils and neutrophils into the lungs of sensitized monkeys.
- ***S*-Y048** also reduces the numbers of bronchial mucin-containing cells following allergen challenge.

What is the clinical significance

- This is the first evidence for a pathophysiological role for 5-oxo-ETE in allergen-induced pulmonary inflammation.
- The OXE receptor antagonist ***S*-Y048** may serve as a novel therapeutic agent in asthma.

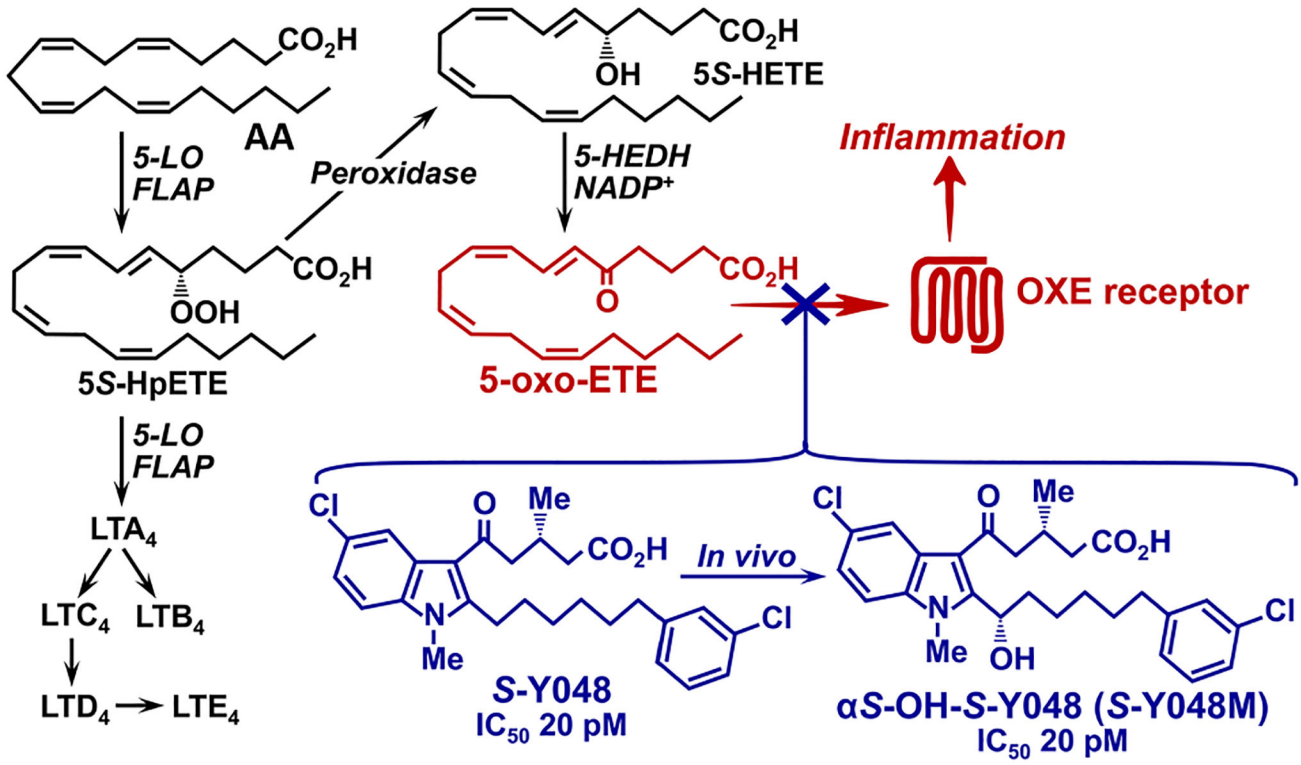

FIGURE 1.

Biosynthesis of 5-oxo-ETE and inhibition of its actions by the OXE receptor antagonist ***S*-Y048**. ***S*-Y048M** is the major plasma metabolite of ***S*-Y048**. 5*S*-HETE, (5*S*-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid); 5*S*-HpETE, (5*S*-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid)

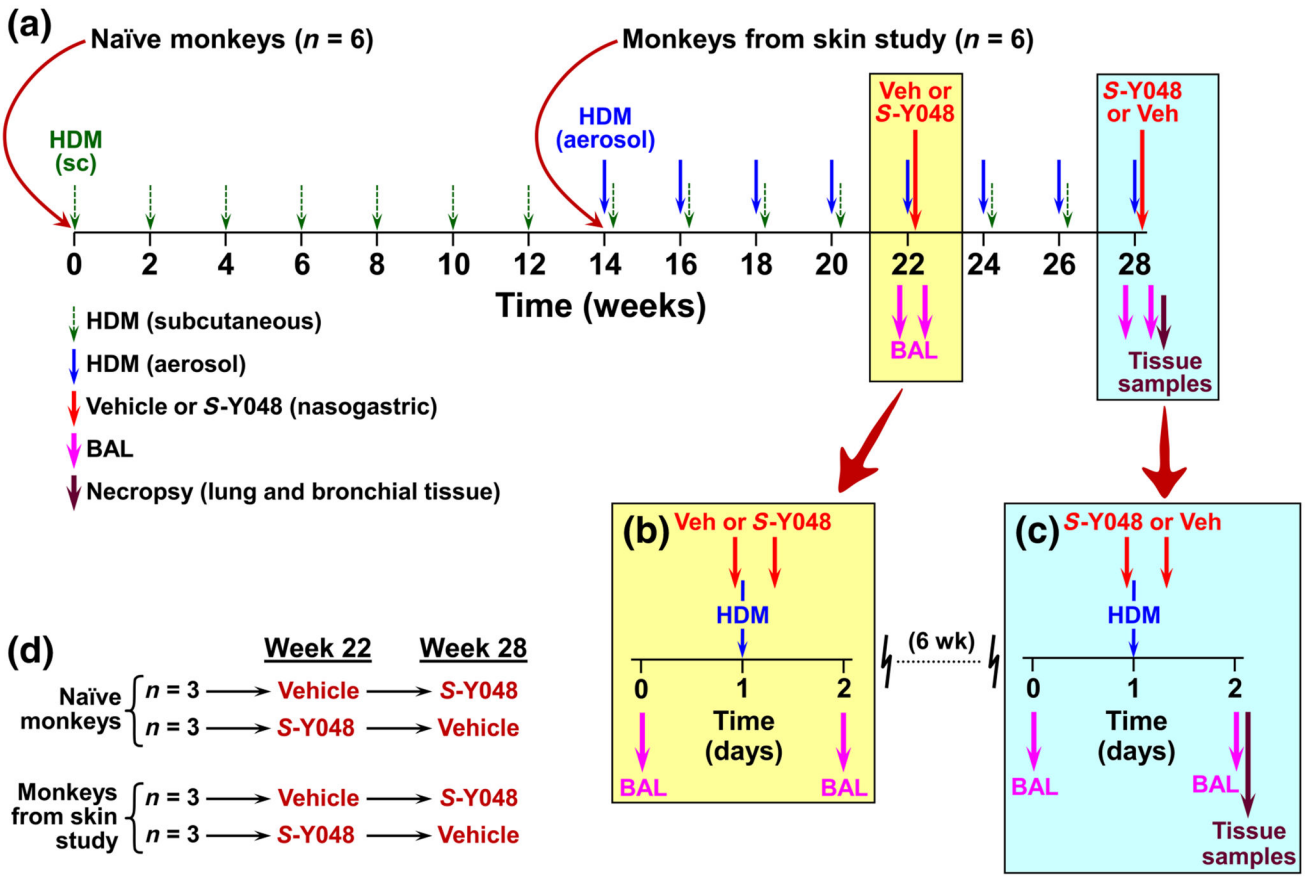

FIGURE 2.

Protocol for evaluation of the effects of ***S*-Y048** on HDM-induced pulmonary inflammation. (a) Animals that had not been used for any other studies ("naïve") were sensitized by a combination of subcutaneous and aerosolized exposures to HDM as described in Section 2.3. Animals from the previous skin study entered the present protocol at the time of first exposure to aerosolized HDM. After sensitization, BAL fluid was obtained 24 h before and 24 h after HDM challenge on Weeks 22 (b) and 28 (c). On both occasions, ***S*-Y048** (2×10 mg·kg^{-1}) or vehicle were administered to monkeys 1 h before and 7 h after exposure to aerosolized HDM. After the final BAL procedure on Week 28, the animals were euthanized, and tissue sections taken at necropsy (c). (d) Half of the naïve monkeys and half of the monkeys from the skin study received vehicle on Week 22 and ***S*-Y048** on Week 28, whereas the other half from each group received ***S*-Y048** on Week 22 and vehicle on Week 28

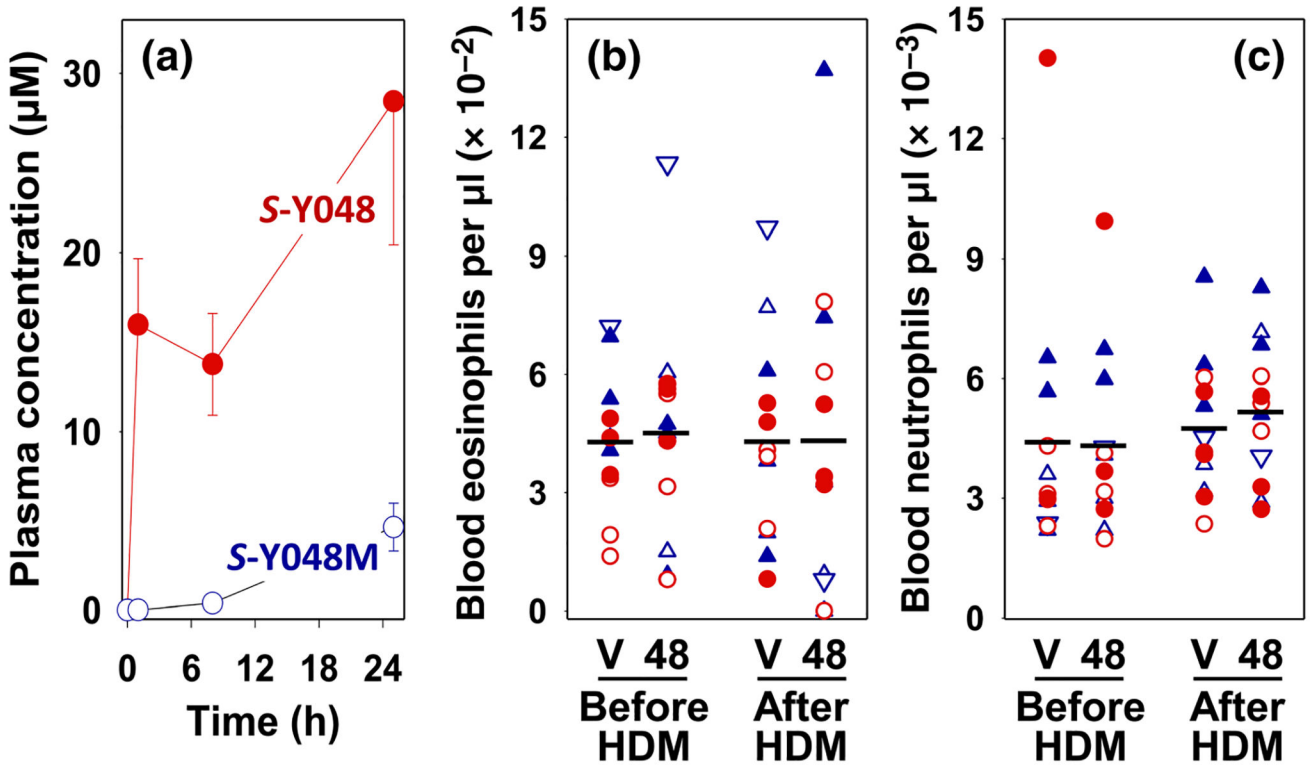

FIGURE 3.

Levels of ***S*-Y048** and numbers of neutrophils and eosinophils in the blood before and after challenge with HDM. (a) Plasma levels (means ± SEM) of ***S*-Y048** (●) and its major plasma metabolite ***S*-Y048M** (○). Blood samples were taken immediately before HDM exposure (1 h after administration of ***S*-Y048**), immediately before the second dose of ***S*-Y048**, and 24 h after HDM exposure. (b and c) Numbers of eosinophils (b) and neutrophils (c) in the blood of individual animals 24 h before and 24 h after challenge with HDM following treatment with either vehicle (**V**) or ***S*-Y048** (**48**). The symbols in panels (b) and (c) represent the following subgroups: Δ, naïve animals that received vehicle first and ***S*-Y048** 6 weeks later; ∇, outlier ***a*** in Figure 4g (which was a naïve animal that received vehicle first); ▲, naïve animals that received ***S*-Y048** first; ○, animals from the skin study that received vehicle first; ●, animals from the skin study that received ***S*-Y048** first. Data from all 12 monkeys are included in all of the panels

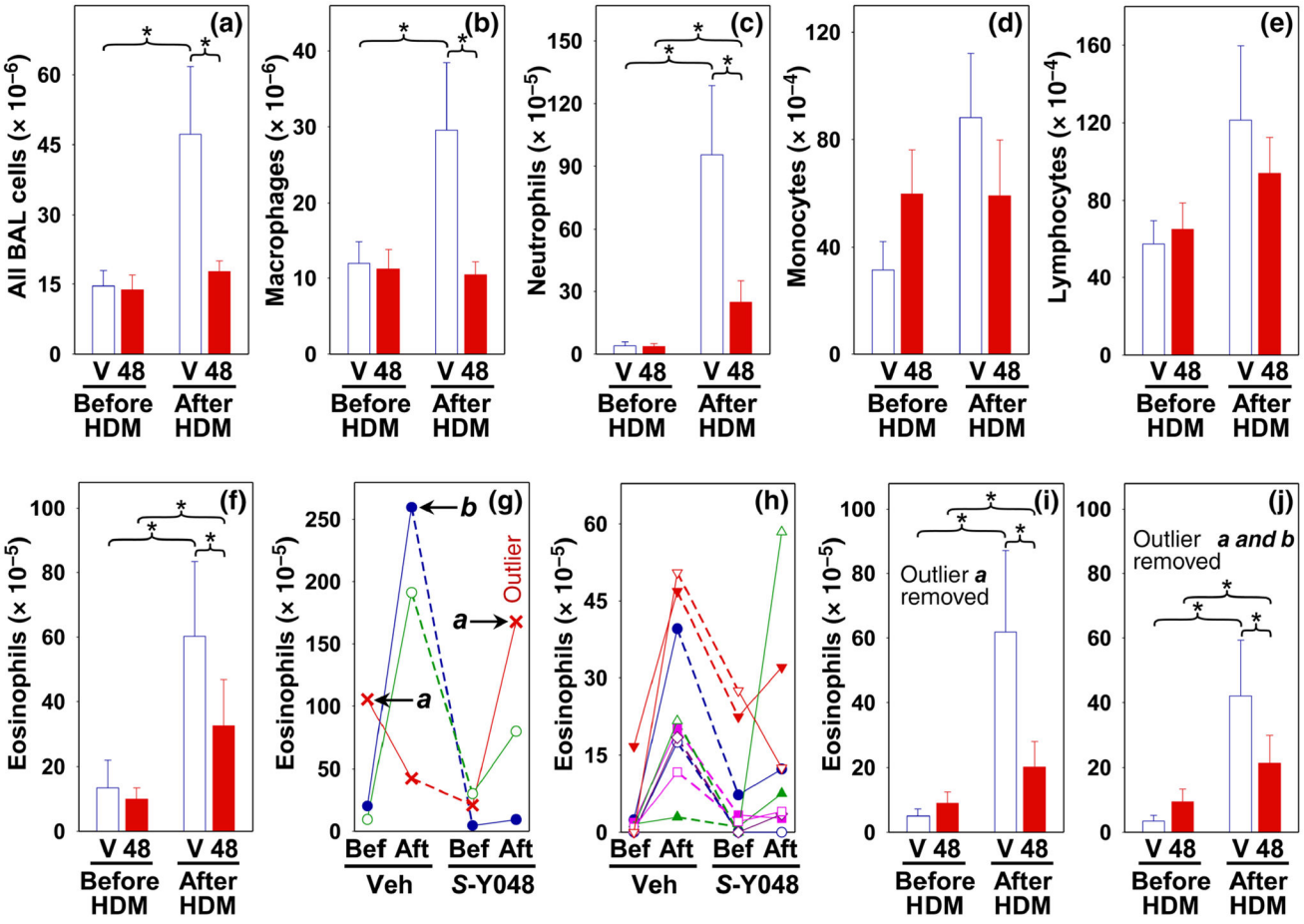

FIGURE 4.

Effects of ***S*-Y048** on the numbers of inflammatory cells in BAL fluid after challenge with HDM. BAL was performed 24 h before (before HDM) and 24 h after (after HDM) challenge with HDM. Vehicle (**V**; open bars) or ***S*-Y048** (**48**; closed bars) at a dose of 10 mg·kg^{-1} was administered by nasogastric intubation 1 h before and 7 h after aerosolized HDM. Values are the means ± SEM of the total numbers of all cells (a), macrophages (b), neutrophils (c), monocytes (d), lymphocytes (e), and eosinophils (f) in BAL fluid. (g and h) Numbers of eosinophils in BAL fluid from each of the 12 animals in the study. The animals are divided into two groups for illustrative purposes only, with the three highest responders to HDM shown in (g) and lower responders shown in (h). Different animals are designated by different symbols. The arrows in panel (g) show the data points that were found to be outliers following analysis using Grubbs' outlier test. "X" symbols show all of the BAL eosinophil data for outlier **a**. Shown for each animal, from left to right: before (**Bef**) and after (**Aft**) HDM for animals treated with vehicle (**Veh**) or ***S*-Y048**. (i) Mean numbers of eosinophils in BAL fluid after removal of the data for outlier ***a***. (i.e., $n = 11$). (j) Mean numbers of eosinophils in BAL fluid after removal of the data for both outliers ***a*** and ***b***. (i.e., $n = 10$). The data shown in all of the bar graphs were not normally distributed and were analysed using Friedman repeated measures analysis of variance on ranks for paired data with the Student-Newman–Keuls method as a post hoc test. All values except for panels (g) and (h) are means ± SEM. *$P < 0.05$

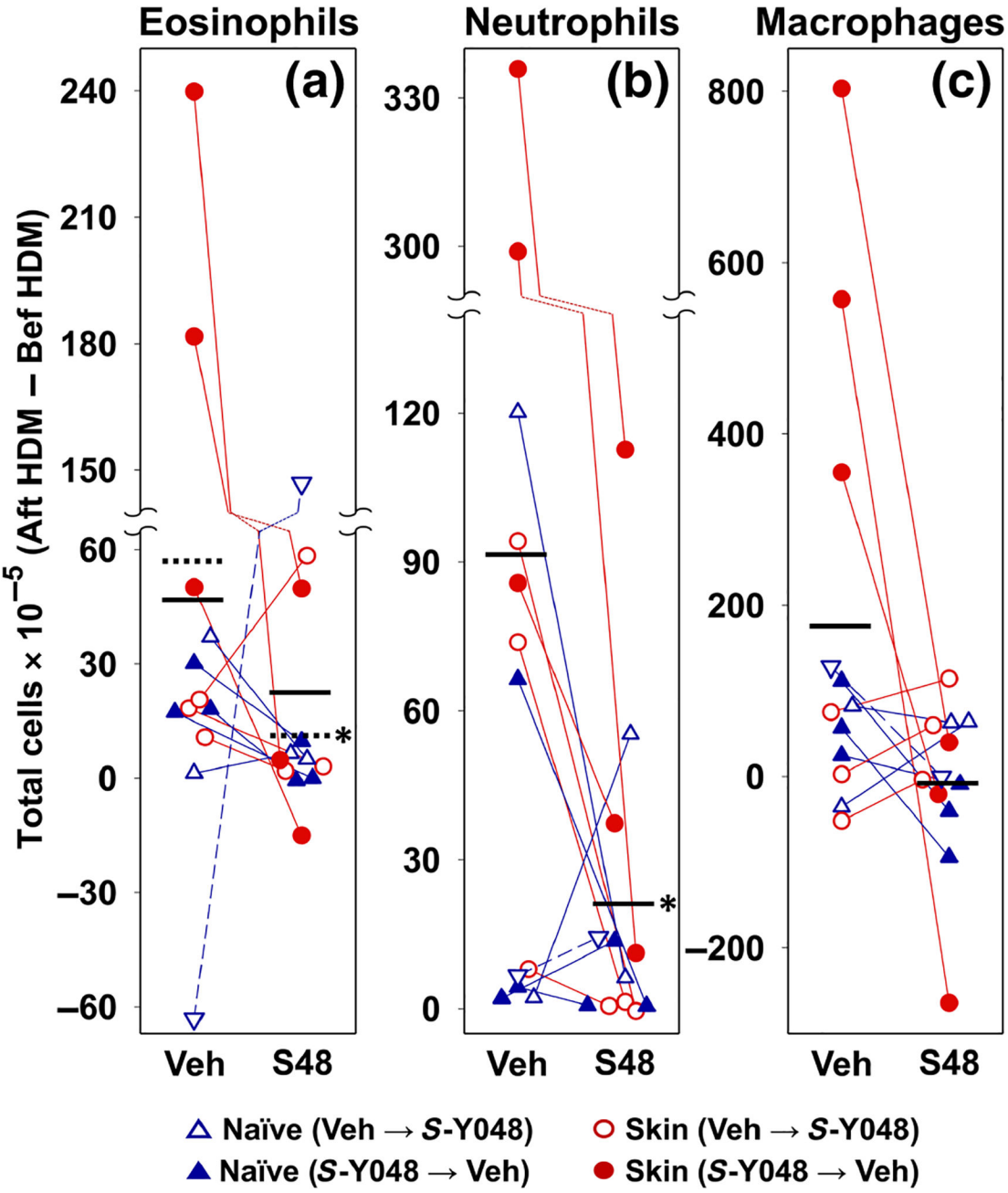

FIGURE 5.

Effects of ***S*-Y048** on HDM-induced increases in BAL inflammatory cells for individual monkeys. Each symbol represents the increase in the number of eosinophils (a), neutrophils (b), or macrophages (c) resulting from challenge with aerosolized HDM (i.e., BAL cell number 24 h after challenge minus BAL cell number before challenge). The symbols represent the following subgroups: Δ, naïve animals that received vehicle first and ***S*-Y048** 6 weeks later; ∇, outlier ***a*** in Figure 4g (which was a naïve animal that received vehicle first); ▲, naïve animals that received ***S*-Y048** first; ○, animals from the skin study that received vehicle first; ●, animals from the skin study that received ***S*-Y048** first. The means

(n = 12) are shown with solid horizontal lines. In panel (a), the means excluding outlier ***a*** (n = 11) are shown with dotted lines. The data for eosinophils and macrophages were not normally distributed, in which case the Wilcoxon signed rank test for paired data was used. The neutrophil data were normally distributed, in which case a paired t test was used. *P < 0.05

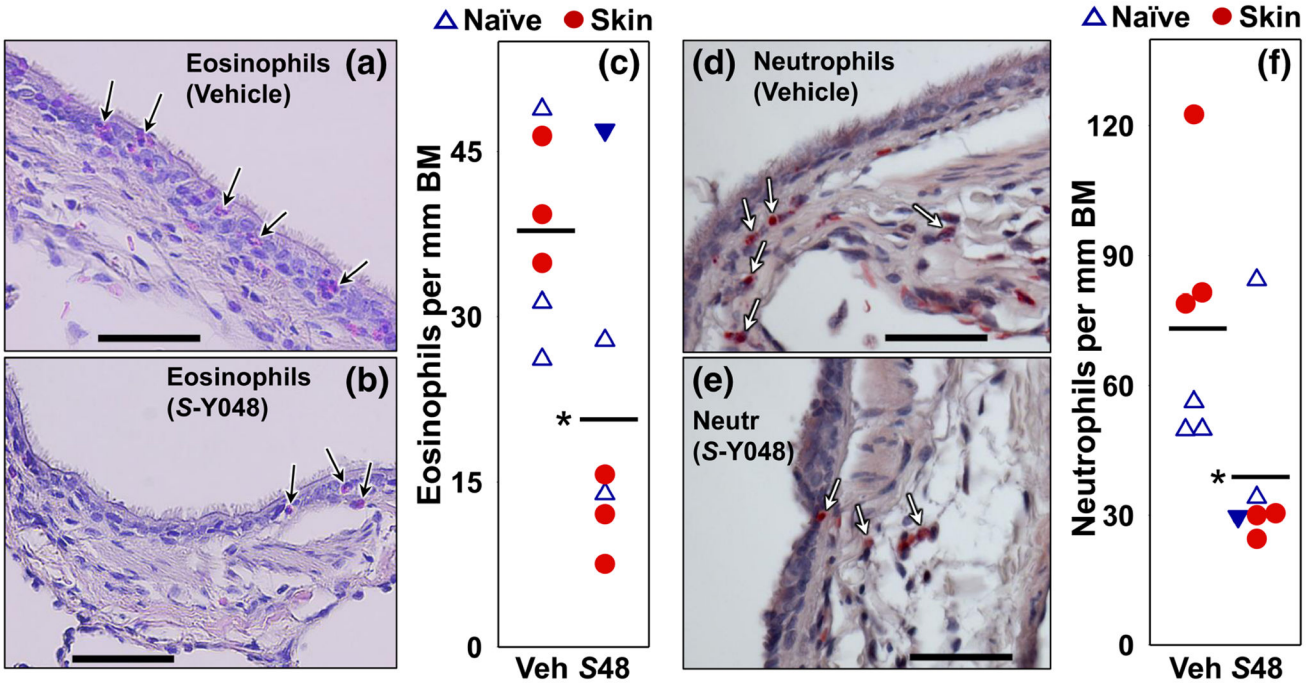

FIGURE 6.

Effect of ***S*-Y048** on lung tissue eosinophils and neutrophils following HDM challenge. Lung sections from monkeys challenged with HDM in the presence of either vehicle (a, d) or ***S*-Y048** (b, e) were stained with either haematoxylin and eosin to reveal eosinophils. (a, b) or with naphthol AS-D chloroacetate and New Fuchsin to reveal neutrophils (d, e). Examples of positively stained cells are indicated with arrows. Original magnification, 400×; scale bars, 50 μm. The numbers of eosinophils (c) and neutrophils (f) per millimetre basement membrane are shown for sections from each of the animals after HDM challenge in the presence of either vehicle (**Veh**) or ***S*-Y048** (**S48**). The mean values for the six animals in each group are indicated by horizontal lines. ●, Monkeys that were previously used in the skin study; Δ, ▼, naïve monkeys; ▼, outlier ***a*** (Figure 4g). The data were normally distributed and analysed using the Student's *t* test for unpaired data; $*P < 0.05$

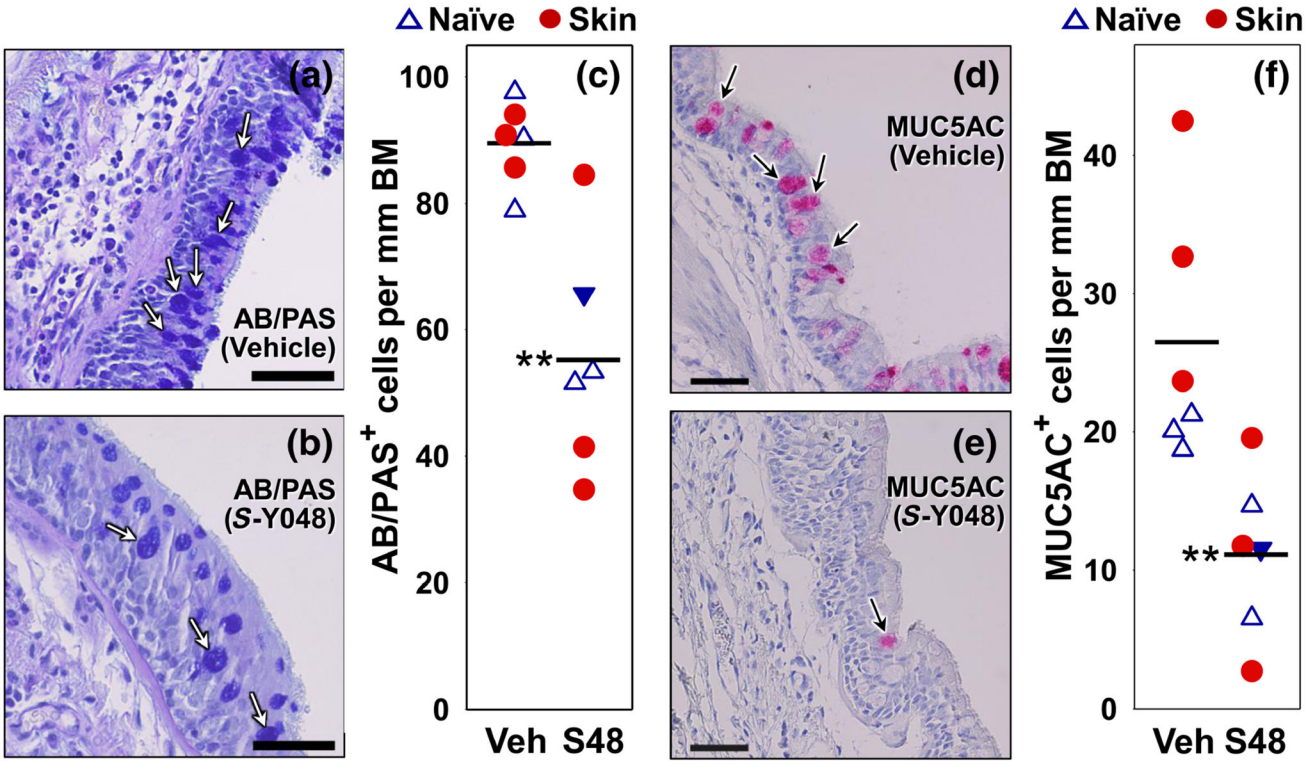

FIGURE 7.

Effect of ***S*-Y048** on the numbers of mucin-containing cells following HDM challenge. Lung sections from monkeys challenged with HDM in the presence of either vehicle (a, d) or ***S*-Y048** (b, e) were stained with AB/PAS (a, b) or immunostained with an antibody against MUC5AC (d, e). Examples of positively stained cells are indicated with arrows. Original magnification, 200×; scale bars, 50 μm. The numbers of cells staining positively for AB/PAS (c) and MUC5AC (f) per millimetre basement membrane are shown for sections from each of the animals after HDM challenge in the presence of either vehicle (**Veh**) or ***S*-Y048** (**S48**). The mean values for the six animals in each group are indicated by horizontal lines. ●, Animals that were previously used in the skin study; Δ, ▼, naïve monkeys; ▼, outlier ***a*** (Figure 4g). The data were normally distributed and analysed using the Student's *t* test for unpaired data; ** $P < 0.05$