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## **Effective Antigen Presentation to Helper T Cells by Human Eosinophils**

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**Abbreviations:**

HDM , house dust mite extract; TG, Timothy grass extract; APC, antigen presenting cell; DC, dendritic cell; Th, helper T; MHC, major histocompatibility complex; HLA, human leukocyte antigen; mAb, monoclonal antibody; PPD, purified protein derivative; SEB, staphylococcal enterotoxin B

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**SUMMARY**

Although eosinophils are inflammatory cells, there is increasing attention on their immunomodulatory roles. For example, murine eosinophils can present antigen to CD4<sup>+</sup> T helper (Th) cells, but it remains unclear whether human eosinophils also have this ability. This study determined whether human eosinophils present a range of antigens, including allergens, to activate Th cells, and characterized their expression of MHC class II and co-stimulatory molecules required for effective presentation. Human peripheral blood eosinophils purified from non-allergic donors were pulsed with the antigens house dust mite extract (HDM), Timothy Grass extract (TG), or

*Mycobacterium tuberculosis* purified protein derivative (PPD), before co-culture with autologous CD4<sup>+</sup> Th cells. Proliferative and cytokine responses were measured, with eosinophil expression of HLA-DR/DP/DQ and the co-stimulatory molecules CD40, CD80 and CD86 determined by flow cytometry. Eosinophils pulsed with HDM, TG or PPD drove Th proliferation, with the response strength dependent on antigen concentration. The cytokine responses varied with donor and antigen, and were not biased towards any particular Th subset, often including combinations of pro- and anti-inflammatory cytokines. Eosinophils upregulated surface expression of HLA-DR/DP/DQ, CD80, CD86 and CD40 in culture, increases that were sustained over 5 days when incubated with antigens, including HDM, or the major allergens it contains, Der p 1 or Der p 2. Human eosinophils can, therefore, act as effective APC to stimulate varied Th cell responses against a panel of antigens including HDM, TG or PPD, an ability that may help to determine the development of allergic disease.

## INTRODUCTION

Eosinophil involvement in inflammatory conditions affecting the skin, gastrointestinal tract and upper and lower airways is well-documented.<sup>1,2</sup> In addition to their role as degranulating effector cells, more recent findings emphasize the immunomodulatory properties of eosinophils,<sup>3</sup> and other important effector functions such as a potential role in maintaining host survival in life-threatening respiratory viral infections.<sup>4</sup> One question that has attracted interest is whether eosinophils can modulate immune responses by acting as antigen presenting cells (APC) to stimulate CD4<sup>+</sup> helper T (Th) cell responses. It has been known for many years that *in vitro* culture of eosinophils

with GM-CSF, typically added to prevent their apoptosis, can also induce expression of MHC Class II,<sup>5</sup> which could equip them for antigen presentation.

Consistent with APC function, murine eosinophils home to lymphoid tissue and provide a second signal for T cell activation through the expression of key co-stimulatory molecules such as CD80 and CD86.<sup>6,7</sup> Although it has been demonstrated that human eosinophils can express CD86 when taken from hyper-eosinophilic patients,<sup>8</sup> or stimulated with IL-3,<sup>9</sup> it is unclear how commonly, or in what circumstances, they display such co-stimulatory molecules. There are also reports that human eosinophils can process and present antigen to activate specific T cells,<sup>10</sup> but, again, it remains to be established how widespread is such ability in different individuals and for different antigens. Despite the evidence that eosinophils have the potential to act as APC to drive Th cell responses and thereby propagate inflammation,<sup>11-13</sup> other findings have suggested that this is limited to super-antigens and peptides rather than proteins that require processing.<sup>6,14</sup>

The effects of Th activation are critically dependent on the subset(s) that respond, and the associated cytokines they produce. In type I hypersensitivity responses Th2 cells produce cytokines, such as IL-5 and IL-13,<sup>15</sup> that promote eosinophil activation and proliferation,<sup>16</sup> but in healthy donors the response to common allergens such as Timothy grass<sup>17</sup> or house dust mite<sup>18</sup> is associated with Th cells producing both Th1 and Th2 cytokines. However it is not known whether eosinophil antigen presentation preferentially supports responses by any particular Th type.

To address the unanswered questions about their roles as APC, we performed a comprehensive examination of the ability of purified peripheral blood human eosinophils to present a variety of protein antigens to autologous CD4<sup>+</sup> Th cells. The focus here was on donors with no history of allergy, since we wished to establish the ability of eosinophils to contribute to helper activation in the absence of any pre-existing strong pathogenic Th2 response. This work is possible because healthy donors harbor allergen specific Th cells.<sup>19,20</sup> Helper responsiveness was tested to eosinophils pulsed with the allergens house dust mite extract (HDM), Timothy Grass extract (TG), Der p 1, Der p 2, or the microbial recall antigen *Mycobacterium tuberculosis* purified protein derivative (PPD), with eosinophil expression of MHC Class II molecules and the co-stimulatory molecules CD40, CD80 and CD86 characterized. It was also determined whether any Th cytokines elicited by eosinophil antigen presentation exhibited a bias towards particular effector or regulatory subsets.

## **MATERIALS AND METHODS**

### **Materials**

CD-16 immunomagnetic beads, the Human CD4<sup>+</sup>T Cell Isolation Kit II and magnetically activated separation columns were from Miltenyi Biotec (Surrey, UK). HDM (*Dermatophagoides pteronyssinus*) and TG extracts, (both certified LPS free and obtained from NIBSC, UK) were dialyzed using slide-A- Lyzer dialysis cassettes (Thermo scientific, UK) for 24 hours and used at a final concentrations of 500—2500 IU/ml. PPD (Satens Serum Institute, Denmark) was added to cell cultures at a final concentration of 5 µg/ml. *Dermatophagoides pteronyssinus* allergens, Der p 1 or Der p 2 (Indoor Biotechnologies Ltd) were used at final concentrations 10 µg/ml. All cells

were cultured in RPMI 1640 (Labtech International Ltd, UK) supplemented with HEPES and 100 IU/ml penicillin, 100 µg/ml streptomycin, L- glutamine 5% (v/v) (Gibco, Life Technologies, Paisley, UK) and 5% (v/v) heat-inactivated autologous serum, with 10nM granulocyte-macrophage colony-stimulating factor (rhGM-CSF, R&D systems, Abingdon, UK). The latter was essential to prevent eosinophil apoptosis during co-culture. The following mAb were used in these studies: HLA-DR/DP/DQ (clone TL2.1), CD1a (clone H1149), CD40 (clone5 C3), CD80 (L307.4) and CD86 (clone 2331) were all from BD Pharmingen, Oxford, UK. Siglec-8 mAb (clone 7CP) was from Biolegend, London, UK. Cytokine levels were measured by Multiplex array for IFN- $\gamma$ , IL-3, IL-6, IL-10, IL-17A and TNF- $\alpha$  (Luminex, Millipore, Watford, UK) and by ELISA for IL-9 and IL-13 (Biolegend).

### **Eosinophil and Th cell Isolation**

Human eosinophils and CD4<sup>+</sup> Th cells were purified from individuals with no clinical history of allergy or eosinophilia ( $\leq 0.5 \times 10^6$  eosinophils/ml), and who were not taking any medication for allergic disease. The inclusion of patients with hemochromatosis, who were being routinely bled to treat the disease, allowed relatively large numbers of eosinophils for some experiments to be collected from whole units of blood in the face of normal eosinophil counts. All subjects gave informed consent and the study was approved by the North of Scotland Research Ethics Service (ref 09/S0801/16). Eosinophils were purified from samples of peripheral blood using our standard technique<sup>13</sup> using dextran sedimentation and centrifugation on Percoll gradients followed by CD16-dependent negative immunomagnetic selection. To obtain CD4<sup>+</sup> Th cells, peripheral blood mononuclear cells (PBMC) were isolated by density gradient

centrifugation<sup>21</sup> and non-target cells depleted by negative selection following the manufacturer's instructions.

Using these methods, eosinophils and Th cells with respective purities of at least 98% were obtained, as expected from previous reports.<sup>22</sup> In particular, the eosinophil preparations were free from any detectable cells expressing the CD1a, CD14 or CD19 markers for professional APC types (Fig. S1. see supplementary materials). All cell preparations exhibited greater than 98% viability as assessed by trypan blue exclusion.

### **Cell cultures**

Eosinophils ( $5 \times 10^5$ /ml) were incubated in culture medium for up to 5 days in the presence of rhGM-CSF (10 nM) to inhibit their spontaneous apoptosis as previously described,<sup>23</sup> with or without the addition of antigens. When co-cultured with autologous CD4<sup>+</sup> Th cells, eosinophils were first pulsed with antigens by overnight incubation, then washed and added at  $5 \times 10^5$ /ml in medium containing GM-CSF to the Th cells ( $1 \times 10^6$ /ml) for 5 days; these conditions were found to give optimal Th responses in pilot experiments (data not shown). Cultures of autologous PBMC ( $1.25 \times 10^6$ /ml), with or without antigen, provided controls for comparison of Th responsiveness.

### **Th cell responses**

Proliferative Th cell responses were determined by incorporation of <sup>3</sup>H-thymidine in triplicate 100 $\mu$ l volumes withdrawn from cultures 5 days after stimulation as previously described, with results presented as CPM.<sup>24</sup> Cytokine levels in cultures were measured

by bead array for IFN- $\gamma$ , IL-3, IL-6, IL-10, IL-17A and TNF- $\alpha$  and by ELISA for IL-9 and IL-13, according to the manufacturers' instructions. Cytokine responses >2x background in unstimulated wells were considered significant.<sup>21</sup> To test the dependency of responses on MHC class II<sup>25</sup>, antigen-pulsed eosinophils were incubated with blocking antibody before co-culture with autologous CD4<sup>+</sup> Th cells.

### **Immunostaining and flow cytometry**

Cell surface expression markers were examined using flow cytometry for HLA-DR,-DP,-DQ (fluorescein isothiocyanate), and co-stimulatory molecules, CD40 (BD Horizon V450), CD80 (Alexa Fluor 700), and CD86 (allophycocyanin) using established protocols.<sup>13</sup> Briefly, eosinophils and CD4<sup>+</sup> Th cells were removed from co-culture, washed and saturating quantities of primary antibodies or specific isotype controls were added to the cells and incubated for 40 min at 4°C in the dark, washed and fixed. Human dendritic cells (DC) were identified by staining cells with CD1a (allophycocyanin) while eosinophils were identified by staining with a specific marker, siglec-8 (phycoerythrin).<sup>26</sup> An EBV-transformed B cell line was used as a source of cells that stained positively for HLA-DR/DP/DQ, CD40, CD80 or CD86 (Fig. S2, see supplementary material). Multiple panels of conjugated antibodies were used to identify the subpopulation of immune cells and the corresponding specific cell surface markers. Ten thousand events were collected on flow cytometer, LSRII (BD Biosciences) using FACS Diva software (BD Biosciences). Analysis was performed using FlowJo software (Treestar, Inc., Ashland, Oreg. USA). In all plots, dead cells, cellular debris and cell aggregates were excluded during the gating process.



## Statistics

Data were analyzed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA), and since D'Agostino-Person Omnibus and Shapiro-Wilk normality tests were failed, non-parametric methods were used (Wilcoxon-Signed-Rank-test or Mann-Whitney U Test as appropriate). Bonferroni corrections were applied to multiple comparisons. Data were considered to be statically significant if  $p < 0.05$ , and are expressed as either median and interquartile range (IQR), or individual data points if  $n < 4$ .

## RESULTS

### *Eosinophils pulsed with antigen induce proliferative Th responses*

We first examined the ability of human eosinophils pulsed with a range of concentrations of the allergens HDM or TG, or with our standard concentration of the control microbial recall antigen PPD, to induce proliferation by autologous CD4<sup>+</sup> Th cells after 5 days of co-culture. In a series of 9 experiments, significant ( $p < 0.001$ ) increases in proliferation were observed when eosinophils had been pulsed by pre-incubation with the allergens HDM (Fig. 1a) or TG (Fig. 1b) at final concentrations of 1000, 1500 or 2500 IU/ml, compared with medium alone. The strongest responses were induced by HDM or TG at 2500 IU, and this concentration of the allergens was therefore used in all subsequent experiments. Eosinophils pulsed with the control antigen PPD also elicited significant proliferation at the standard concentration of 5  $\mu\text{g/ml}$  (Fig. 1c). To confirm that responses to each stimulus required the presence of both eosinophils and Th cells, proliferation was compared in cultures containing each cell type alone or together, with or without antigen pulsing (Fig. 2). Proliferative responses were significant only when both antigen-pulsed eosinophils and Th cells were

added, and, strikingly, these responses were similar in size to those seen when unfractionated PBMC were stimulated with the respective antigen as a positive control. It should be noted that the ability of the eosinophil preparations to drive such strong Th cell responses cannot be explained by the presence of contaminating APC, since none could be detected by flow cytometry (Fig. S1, see supplementary material). Control analyses also confirmed that proliferative responses in antigen-stimulated co-cultures were mediated by Th cells, but not eosinophils (median CD4<sup>+</sup> Th cell count increased by 30% in HDM stimulated co-cultures after 5 days p=0.016, Wilcoxon signed rank test, n=8, versus no increase in median eosinophil numbers). The ability of Th cells to respond to antigen-pulsed eosinophils was MHC class II dependent, since incubating HDM-pulsed eosinophils with blocking mAb specific for HLA-DR/DP/DQ<sup>25</sup> significantly (P<0.001) reduced T cell proliferation by 77% (Fig. S3, see supplementary material).

#### ***Eosinophil expression of MHC class II and co-stimulatory molecules***

Effective APC require expression of both MHC class II and co-stimulatory molecules, so we next tested whether cultured eosinophils display HLA-DR/DP/DQ and CD40, CD80 and CD86. Examples of flow cytometric analyses, and graphical summaries of data from 9 independent experiments, demonstrate eosinophil expression of HLA-DR/DP/DQ (Fig. 3) and CD40, CD80 and CD86 (Fig. 4) during 5 day cultures, with or without HDM addition. It can be seen that, within 24 hours, eosinophil expression of MHC class II, and the co-stimulatory molecules CD40, CD80 and CD86, was significantly increased in all cultures, an effect that may be at least partly due to the presence of GM-CSF added to the medium to prevent eosinophil apoptosis.<sup>5</sup> However,

there was a further effect of HDM. Addition of the antigen sustained the elevated levels of MHC class II, CD40, CD80 and CD86 over the course of the incubation, since, without HDM, expression of all the markers fell back by day 5 to levels not significantly above those seen at the beginning of the culture.

The HDM preparation is a simple extract, so we next tested whether the major allergens it contains, Der p 1 and Der p 2, recapitulate its ability to sustain enhanced expression of MHC class II and co-stimulatory molecules by eosinophils. Eosinophils incubated with purified Der p 1 or Der p 2 exhibited similar sustained increases in expression of HLA-DR\DP\DQ, CD40, CD80 and CD86 compared with those elicited by the crude HDM preparation (Fig. 5).

#### ***Cytokine responses induced by eosinophils pulsed with antigen***

Having demonstrated the ability of eosinophils to present antigen to drive Th proliferative responses, the question arises as to whether pro- or anti-inflammatory cytokines, or cytokines associated with any particular CD4<sup>+</sup> subset are produced. Signature cytokines for the major subsets Th1 (IFN- $\gamma$ ), Th2 (IL-13), Th9 (IL-9), Treg (IL-10), Th17 (IL-17A) and the inflammatory cytokines TNF- $\alpha$ , IL-3 and IL-6, were measured in co-cultures of CD4<sup>+</sup> Th cells and eosinophils with, or without, pulsing with the antigens HDM, TG or PPD. Different patterns of cytokine response to the antigens were seen in cultures from each of the donors tested (n=6), with examples illustrated in Figure 6, and all results summarized in Table 1. It can be seen that antigen-pulsed eosinophils are capable of eliciting a wide range of cytokines tested. Although IL-6 production was the most frequently seen response, cultures of antigen-pulsed

eosinophils could also contain complex mixtures of both pro- and anti-inflammatory cytokines, and, overall, there was no clear or consistent bias towards any particular cytokine type.

## DISCUSSION

The present study has established the ability of human eosinophils to present a wide variety of protein antigens, including allergens, to stimulate proliferative and cytokine responses by CD4<sup>+</sup> Th cells. In line with their ability to elicit responses to antigen, eosinophils exhibited upregulated MHC class II and costimulatory molecules in culture. Since the donors tested here had no clinical history of allergy, the results raise the possibility that eosinophils acting as APC can help determine whether Th responses to allergen are elicited or become pathogenic.

There is now a substantial body of evidence demonstrating that murine eosinophils presenting antigen have the ability to stimulate Th cells, including the induction of primary responses, and that they act as true professional APC in homing to lymph nodes, where they can interact with Th cells.<sup>12</sup> We show here, for the first time, that human eosinophils are also effective APC for a variety of protein antigens *in vitro*, able to evoke significant Th proliferative responses comparable in magnitude to those seen when paired PMBC samples are challenged with the same antigen. Thus, human eosinophil presentation to Th cells is limited neither to peptide antigens nor superantigens that do not require processing, as has been suggested,<sup>6,14</sup> but may instead contribute to responses against many different proteins. The question arises as to why previous studies have not clearly demonstrated such APC activity by human

eosinophils, particularly when this ability would be predicted from murine studies.<sup>6,7</sup> While it is not possible to pinpoint with any confidence which factor, or combination of factors, accounts for the great sensitivity of our study versus those carried out several years ago in other laboratories, we can offer a number of suggestions. Our cultures are optimised to support proliferative responses by antigen specific T cells present at low precursor frequency, with relatively long time courses allowed for responses to develop, and supplementation with autologous serum to enhance sensitivity, rather the foetal calf serum used elsewhere. We also exploited polyclonal T cells as detectors of antigen presentation by eosinophils, rather than rely on T cell clones raised against other APC types. Whatever the explanation, whether the ability we describe of human eosinophils to act as APC *in vitro* is replicated *in vivo*, or in patients with hypersensitivity, now needs to be established. It is noteworthy that upregulation of eosinophil MHC Class II expression observed in asthma<sup>27</sup>, chronic eosinophilic pneumonia<sup>28</sup>, and eosinophilic esophagitis<sup>29</sup> is consistent with such an immune modulatory role.<sup>10,12</sup>

APC function is dependent on display of MHC class II and costimulatory molecules.<sup>30</sup> We not only confirm that cultured human eosinophils express HLA-DR/DP/DQ, but also show expression of all three major co-stimulatory molecules, CD40, CD80 and CD86 by these cells. Eosinophils in culture require addition of GM-CSF to prevent apoptosis, and the presence of this cytokine is likely to have contributed to the APC phenotype seen here, since eosinophils purified from the spleens of IL-5 transgenic mice were also observed to express MHC Class II, CD40, CD80 and CD86 when stimulated with GM-CSF.<sup>10</sup> Previous studies of human eosinophils have described upregulation of MHC class II and CD86 in response to cytokine or super-antigen

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exposure,<sup>9,28,31</sup> but, to our knowledge, we are the first to report expression of such a complete APC surface phenotype in cultured human eosinophils. Although it could be argued that the precise conditions *in vitro* do not reflect those *in vivo*, the results nevertheless establish that eosinophils have the potential to present antigen very effectively, and the induction of APC function by stimuli such as GM-CSF *in vivo* may well represent an important mechanism by which eosinophils influence immune responses to allergens. In addition, GM-CSF was not the only factor upregulating APC surface markers, since we demonstrated that the increases in the expression of HLA-DR/DP/DQ, CD40, CD80 and CD86 were sustained for up to 5 days of co-culture by eosinophils stimulated with whole HDM extract or the major HDM allergens Der p 1 and Der p 2. A number of studies have identified similar effects of HDM extract, Der p 1 or Der p 2 on other cell types. For example, Der p 1 stimulation of monocyte-derived DC isolated from donors allergic to HDM increased CD86 expression, while control non-allergic subjects had significant increases in CD80 expression<sup>32</sup>, and another study showed that Der p 1 stimulated human peripheral blood DC to increase expression of HLA-DR, CD80 and CD86.<sup>33</sup> The underlying mechanisms remain to be established, but may include proteolytic activity of the allergen,<sup>34</sup> or its interaction with pattern recognition receptors. Der p 2 in particular has auto-adjuvant properties due to structural and functional homology with MD-2, the lipopolysaccharide-binding component of the Toll-like receptor 4 signaling complex.<sup>35</sup>

The cytokines elicited during responses to antigen play a key role in determining both protection from infection, and immune pathology. Here, eosinophils acting as APC supported production of a wide range of cytokines that differed between individuals and

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antigens, but with no clear preference for any Th response type. However, our study was of cells from donors with no clinical allergic disease, and so the possibility remains open that eosinophils may skew helper responses towards the pathogenic Th2 subset in patients with overt allergy, or a susceptibility to atopic disease. The notion that eosinophils can drive a variety of Th subsets is supported by a previous study, which also tested co-cultures of human peripheral blood eosinophils and autologous CD4<sup>+</sup> Th cells, and demonstrated both Th1 and Th2 cytokine responses to the super-antigen staphylococcal enterotoxin B (SEB). These workers also demonstrated HLA-DR expression by peripheral blood eosinophils isolated from 50% of the subjects, attributed to GM-CSF added to cultures, but stimulation with SEB did not induce eosinophil expression of CD80 or CD86.<sup>36</sup> The reasons for the differences between this result and the present study may well reflect the use of antigens versus polyclonal activator for stimulation. Others have described the effect of stimulation with HDM antigen on eosinophil function. For example, HDM stimulation of eosinophils *in vitro* led to production of IL-9 that may promote a Th2 immune response,<sup>37</sup> but, although we detect this cytokine in some co-cultures of Th cells and antigen-pulsed eosinophils, HDM did not elicit the response more frequently than other antigens, and IL-9 was not associated with any clear Th subset bias.

Taken together, our data demonstrate that human eosinophils can act as effective APC to stimulate Th responses against a variety of antigens, including the allergens HDM or TG: a property that may contribute to the regulation of responses *in vivo* and to induction or control of pathology in allergic disease, depending on the cytokines elicited. The findings add to the accumulating evidence that eosinophils possess more

complex immunomodulatory roles in allergic disease than previously suspected. Furthermore, any ability of HDM, Der p 1 or Der p 2 to act not only as antigens, but also to increase eosinophil co-stimulatory molecule expression, may enhance their immunogenicity. Having demonstrated the ability of human eosinophils to present antigen, this study opens up new questions as to how important they are in initiating, skewing, amplifying or regulating allergic responses in patients.

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#### **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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## FIGURE LEGENDS

**Figure 1. Eosinophils pulsed with allergen stimulate Th cell proliferative responses.** Panels show proliferation in co-cultures of peripheral blood CD4<sup>+</sup> Th cells and autologous eosinophils that have been pulsed with the antigens HDM (a), TG (b), or PPD (c). Results are expressed as median CPM and IQR from 9 independent experiments (\*p < 0.01, Mann-Whitney U-test with Bonferroni correction).

**Figure 2. Eosinophils pulsed with antigen stimulate Th proliferation comparable to PBMC responses.** Panels show proliferation in cultures containing peripheral blood CD4<sup>+</sup> Th cells and autologous eosinophils, either alone or together, with or without

pulsing of the eosinophils with the antigens HDM (a), TG (b) or PPD (c). Proliferation in PBMC cultures, either untreated or antigen stimulated, is included for comparison. Eo = purified eosinophils, T = purified CD4<sup>+</sup> T cells (\*p<0.05, Mann Whitney U-test with Bonferroni correction).

**Figure 3. Cultured eosinophils express MHC class II.** Representative flow cytometric histograms (n=9) demonstrate HLA DR/DP/DQ expression by purified eosinophils incubated with HDM at day 0 (a) and day 5 (b) of culture (solid line = stained cells, dotted line = unstained cells, dashed line = isotype control). The gate indicates the percentage of eosinophils staining positively for HLA-DR/DP/DQ. HLA-DR/DP/DQ expression on unstimulated (c) or HDM stimulated (d) eosinophils over 5 days of culture is summarized in bar charts, with results expressed as median and IQR of % eosinophils positive for HLA DR/DP/DQ staining from 9 independent experiments (\*p < 0.01, Mann Whitney U-test with Bonferroni correction).

**Figure 4. Cultured eosinophils express costimulatory molecules.** Representative flow cytometric histograms (n=9) demonstrate CD40 (a), CD80 (b) and CD86 (c) expression by purified eosinophils incubated with HDM at day 0 (left panels) and day 5 (right panels) of culture (solid line = stained cells, dotted line = unstained cells, dashed line = isotype control). The gate indicates the percentage of eosinophils staining positively for each co-stimulatory molecule. CD40 (d), CD80 (e) and CD86 (f) expression by unstimulated (left panels) or HDM stimulated (right panels) eosinophils over 5 days of culture is summarized in the bar charts, with results expressed as median

and IQR of % eosinophils positive for each marker from 9 independent experiments (\*p < 0.01, Mann Whitney U-test with Bonferroni correction).

**Figure 5. Der P1 and Der P2 antigens share the ability of HDM to sustain eosinophil surface expression of MHC class II and costimulatory molecules.**

Comparison of the effects of incubation with the purified allergens Der p 1 or Der p 2, or the allergen extract HDM, on numbers of eosinophils that express of HLA-DR\DP\DQ (a), CD40 (b), CD80 (c) and CD86 (d) after 5 days of culture. Results are expressed as median and IQR of % eosinophils positive for each marker from 6 independent experiments (\* p < 0.01, Mann-Whitney U-test with Bonferroni correction).

**Figure 6. Production of multiple cytokines in co-cultures of CD4<sup>+</sup> Th cells and antigen-pulsed eosinophils.** Examples are shown (donors 1 and 5, n=6) of different patterns of cytokine secretion by co-cultures of CD4<sup>+</sup> Th cells and eosinophils pulsed with the antigens HDM, TG or PPD.

**Table 1: Cytokine production by co-cultures of Th cells and eosinophils presenting antigen.**

Cytokine	Stimulus	S1	S2	S3	S4	S5	S6
IFN- $\gamma$	HDM	↑	↔	↔	↔	↔	↔
	TG	↔	↔	↔	↔	↑	↔
	PPD	↔	↔	↔	↑	↑	nt
TNF- $\alpha$	HDM	↑	↔	↔	↔	↔	↔
	TG	↔	↔	↔	↔	↔	↔
	PPD	↔	↔	↔	↑	↑	nt
IL-3	HDM	↑	↔	↔	↔	↔	↔
	TG	↔	↔	↔	↔	↔	↔
	PPD	↔	↔	↔	↔	↔	nt
IL-6	HDM	↑	↔	↔	↑	↑	↑
	TG	↔	↔	↔	↑	↑	↑
	PPD	↑	↔	↔	↑	↑	nt
IL-9	HDM	↑	↔	↔	↔	↑	↔
	TG	↔	↔	↑	↔	↑	↔
	PPD	↔	↔	↔	↑	↑	nt
IL-10	HDM	↑	↔	↔	↔	↔	↔
	TG	↔	↔	↔	↔	↑	↔
	PPD	↔	↔	↔	↑	↑	nt
IL-13	HDM	↑	↔	↓	↔	↔	↑
	TG	↑	↑	↔	↑	↔	↑
	PPD	↔	↔	↑	↔	↑	nt
IL-17	HDM	↔	↔	↔	↔	↑	↓
	TG	↔	↔	↔	↔	↑	↔
	PPD	↔	↔	↔	↑	↑	nt

The table shows whether there is an increase (up arrow,  $SI > 2$ ), decrease (down arrow,  $SI < 0.5$ ) or no change (horizontal arrows,  $SI$  range 0.5-2) in cytokine production in Th cell-eosinophil co-cultures when eosinophils are pulsed with HDM, TG or PPD, compared to unpulsed control. nt=not tested

Figure 1

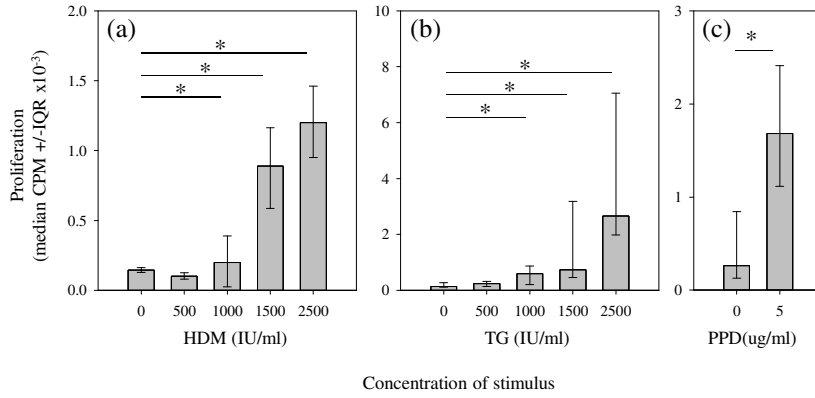


Figure 2

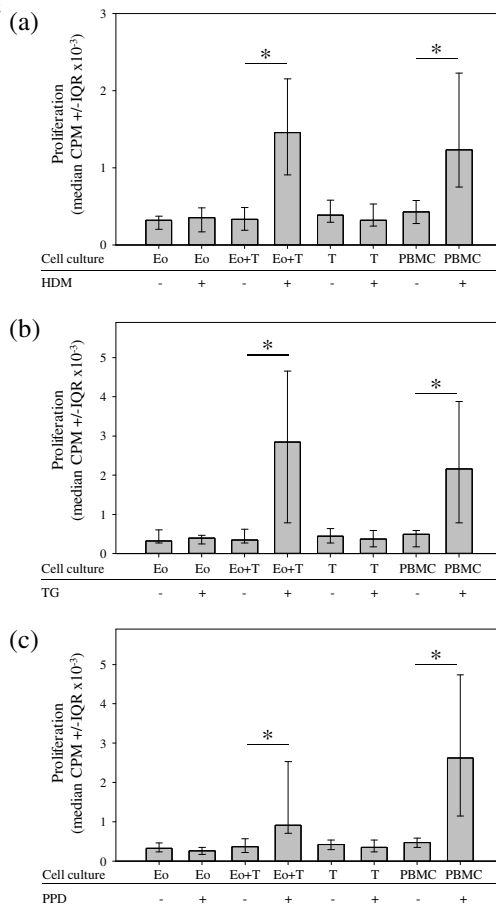


Figure 3

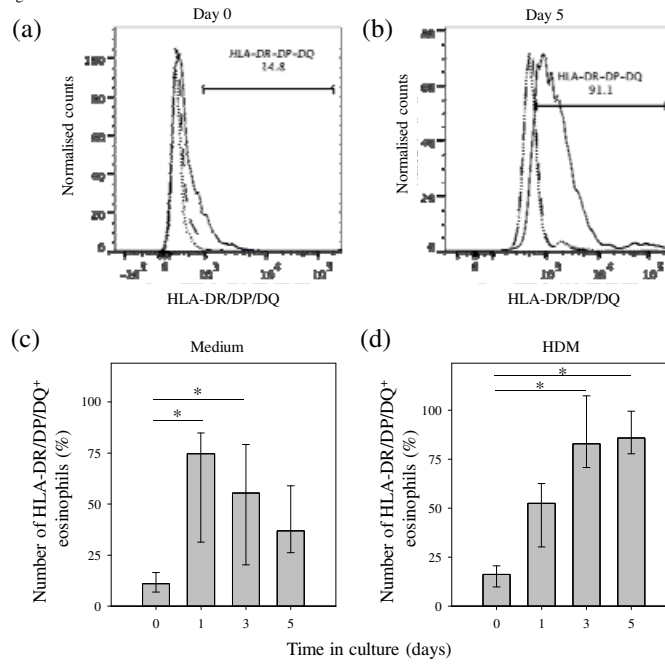


Figure 4

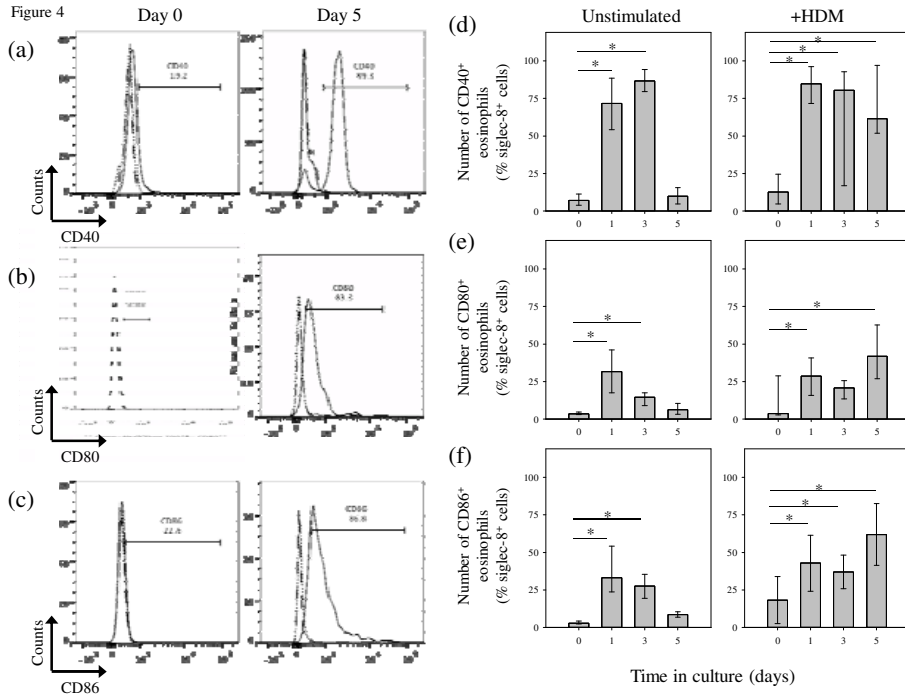




Figure 5

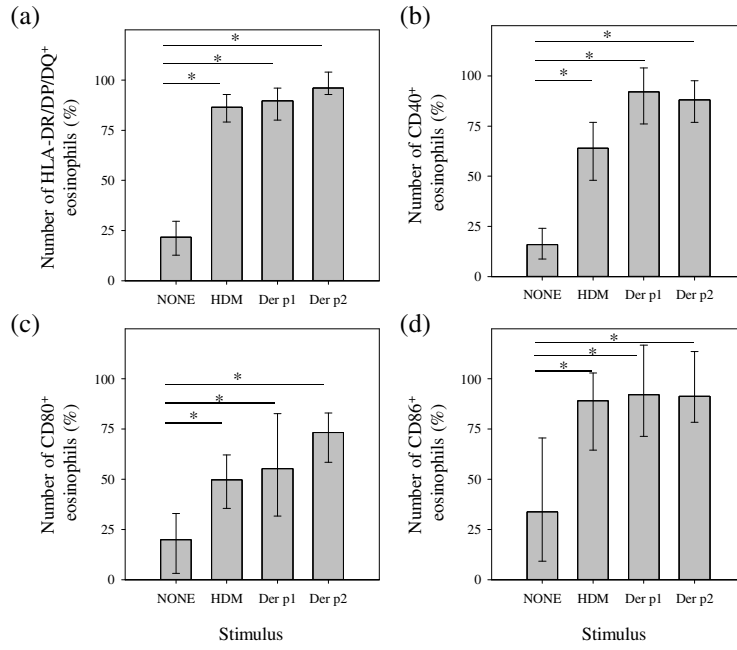


Figure 6

