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Characterization and epitope identification of the T cell response in non-allergic individuals exposed to mouse allergen

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ABSTRACT

Background: Exposure to airborne allergens is a frequent trigger of respiratory allergy and asthma in atopic individuals. While allergic patients suffer hypersensitivity reactions to these allergens, non-allergic individuals do not exhibit clinical symptoms despite environmental exposure to these ubiquitous allergen sources. The aim of this study was to characterize T cell responses in non-allergic laboratory workers, who are heavily exposed to mice allergens (Exposed Non-Allergics, ENA) and compare this data to previously published T cell responses measured in mouse (MO)-allergic patients. METHODS: Peripheral mononuclear cells (PBMC) from ENA subjects were expanded for 2 weeks *in vitro* with mouse urine extract and screened for IFNγ and IL-5 cytokine production in response to mouse antigen-derived peptides by ELISPOT. *Ex vivo* T cell reactivity in the ENA cohort was performed after 6hr stimulation with peptide pools by intracellular staining of CD154.
Results: Vigorous responses were detected, associated with 147 epitopes derived from 16 mouse antigens. As expected, responses in ENA subjects were somewhat lower than those observed in MO-allergics for both responder frequency and overall response magnitude. While responses in allergics were polarized towards IL-5 production and associated with low IFNγ production, ENA responses were not polarized. The composition of targeted antigens and epitopes was overall similar between the two cohorts, with the majority of T cell reactivity directed against Mus m 1 and other major urinary proteins. However, kappa-casein precursor and odorant binding protein Ib were more abundantly recognized in MO-allergics compared to ENA subjects. Additionally, T cell responses against oligopeptides derived from the low molecular weight fraction of mouse urine were also assessed. Interestingly, no difference in the response frequency, magnitude or polarization between MO-allergic and ENA individuals was observed. Finally, assessment of *ex vivo* T cell activation also revealed T cell reactivity in the ENA cohort, with a non-significant trend for lower responses compared to MO-allergics.
Conclusion: Exposure to mouse induces potent T cell responses in non-allergic individuals, targeting similar epitopes as seen in allergic patients.

Introduction

Respiratory allergy is a chronic disease associated with aeroallergens present in the environment and therefore nearly impossible to avoid. For allergic individuals, exposure to these allergens commonly triggers a wide spectrum of allergic symptoms ranging from rhinitis to asthma. Lack of allergic sensitization and allergic symptoms is not due to lack of exposure, since many allergens are ubiquitously found in the environment, and household contacts of allergic individuals, while presumably exposed, are not necessarily sensitized nor do they always display allergic symptoms.

Immune responses from allergic individuals[1] are primarily associated with IgE[2] and type 2 T cell responses.[3] However, in contrast to allergen-specific IgE, which is typically only detected in allergic patients, allergen-specific T cell responses have frequently been reported in non-allergic individuals.[4–6]

Mouse (MO) allergens are of growing importance in children and adults alike as they are potent sensitizers[7] and MO allergies are prevalent, especially in inner city populations.[8,9] Their clinical relevance is underlined by studies indicating that MO-sensitization is a strong correlate of asthma development.[10,11] High IgE titers to MO and to German cockroach have also been associated with atopic dermatitis.[11] Despite their

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Abbreviations

ENA	exposed non-allergic
MO	mouse
PBMC	peripheral blood mononuclear cells
MUP	major urinary protein
MHC	major histocompatibility complex
SFC	spot forming cells

clinical and epidemiological importance, little was known until recently about MO allergens at the molecular level.[12] Mus m 1, a major urinary protein (MUP) from the lipocalin superfamily,[13] defined on the basis of IgE reactivity, is the only allergen listed in the IUIS database.[14]

We recently reported an immunoproteomic approach, that revealed a significant complexity of T cell targets recognized in MO-allergic patients.[15,16] In a first study we identified 106 dominant T cell epitopes from 35 different protein targets recognized by MO-allergic patients.[15] A subsequent study revealed an additional 50 epitopes from mouse urine-derived low molecular weight oligopeptides.[16]

Mouse sensitization has been reported at a prevalence of 10–26%[17,18] in cohorts of animal-care workers, exposed to MO allergens because of occupational duties. Consequently about 74–90% of animal-care workers do not develop allergic systems and are not sensitized to MO allergens, despite heavy exposure. These individuals represent an ideal cohort to study the non-allergic T cell response. The mechanisms by which non-allergic individuals escape sensitization are not fully understood. It is possible that MO allergens are simply not immunogenic for these individuals, which therefore do not develop MO allergen-specific T cell and antibody responses. Alternatively, it is possible that these individuals do develop MO-specific immune responses because of exposure, but the responses are of different quality and quantity as compared to MO allergic donors. The current study addresses these issues.

Methods

Study population and PBMC isolation

A cohort of 19 individuals exposed to mice because of occupational duties in the 3 months preceding the blood donation, but not reporting allergic symptoms (questionnaire based survey) and negative for sensitization for MO allergens, as defined by mouse-specific IgE titers of <0.1 kU_A/L, was studied. These Exposed Non- Allergic individuals are referred to as ENA hereafter. Additionally, for this study data from 14 MO-allergic patients was included from a previous reports.[15,16] These allergic donors exhibited mouse-specific IgE titers of >0.35 (median 2.7 ± 11.42 kU/L) (Table 1). In addition, all allergic donors reported a history of symptoms consistent with mouse allergy, including asthma and rhinitis.

Both cohorts were recruited from San Diego, CA, following Institutional Review Board approval (La Jolla Institute for Allergy and Immunology, La Jolla, CA, IRB protocol no. 112–0217). All patients enrolled in this study provided written consent. Demographic and clinical information is summarized in Table 1. IgE-titers were determined from plasma using the ImmunoCAP (Thermo Fischer, Uppsala, Sweden). PBMCs were isolated from whole blood by density gradient centrifugation according to manufacturers' instructions (Ficoll-Hypaque, Amersham Biosciences, Uppsala, Sweden).

MO urine extract

Mouse urine (mixed gender pooled, unfiltered) was purchased from CliniSciences (Nanterre, France). Low molecular components were removed by filtration centrifugation using Amicon Ultracel tubes with a <3 kDa cut-off filter (Merck Millipore, Darmstadt, Germany). The high molecular weight fraction (>3 kDa) was washed six times with PBS, each time followed by centrifugation at 500×*g* for 10 min in a fresh Amicon Ultracel tube with a 3 kDa cutoff filter. The resulting high molecular weight urine extract was lyophilized and subsequently resuspended in PBS at 20 mg/ml to be used for experiments.

Peptide synthesis and pools

Identification, selection and MHC class II binding prediction of 655 peptides from 1) Mus m 1, 2) Mus m 1-isoforms, 3) mouse proteins homologous to major mammalian allergens and 4) peptides identified by immunoproteomic analysis of the high molecular weight fractions of mouse allergen extracts and conventional allergens (herein referred to as HiMO peptides) was previously described.[15] Additional peptides tested were derived from urinary oligopeptides (low molecular weight fraction of mouse urine) shown to elicit T cell responses in MO-allergic donors in a previous study[16] (herein referred to as LoMO peptides). Peptides were purchased from A and A (San Diego, CA) as crude material on a small (1-mg) scale. Individual peptides were resuspended in DMSO at a final concentration of 40 mg/ml. A complete list of all peptides used in this study is presented in Supplementary Table S1. We previously reported the use of dominant T cell epitopes in the HDM and CR systems, denominated as epitope "megapools".[4,19] Here, the HiMO and was utilized, consisting of 106 dominant epitopes described by Schulten et al., 2018.[15]

Stimulation and expansion of MO-specific T cells with urine MO extracts

For *in vitro* expansion of MO-specific T cells, PBMCs of MO-allergic individuals were stimulated with urine extract prepared as described in the MO urine extract section above (3 μg/ml). Cells were cultured with RPMI 1640 (Omega Scientific) supplemented with 5% human AB serum (Gemini Bioscience), 1% GlutaMAX (Gibco), and 1% penicillin/streptomycin (Omega Scientific) in 24 well plates (BD Bioscience, San Diego, CA) at a density of 2×10^6/ml and incubated at 37 °C. IL-2 (10 U/ml) was added every 3 days after initial stimulation. Cells were harvested on day 14 and screened for IFNγ and IL-5-production by ELISPOT.

Dual ELISPOT assays

The production of IFNγ and IL-5 from cultured PBMCs in response to antigenic stimulation was assessed by dual ELISPOT assays.[20] Cells (1×10^5 cells/well) were stimulated in triplicates with either peptide pools (5 μg/ml) or individual peptides (10 μg/ml), PHA (10 μg/ml), or medium containing 0.25% DMSO (% of DMSO in the pools/peptides) as a control. Spot forming cells (SFC) were counted by computer assisted image analysis (KS-ELISPOT reader, Zeiss, Munich, Germany). Criteria for positivity were ≥20 SFCs for single peptides or ≥100 SFCs for peptide pools per 10^6 PBMCs, $P < 0.05$ based on Poisson test between negative control replicates

Table 1
Description of the cohorts analyzed in this study.

Cohort	Number of donors	Age (Median±SD)	Gender (% of females)	Mouse IgE (kU_A/L) (Median±SD)	Clinical status
ENA	19	30 ± 7	63%	<0.1	Exposed non-allergic individuals
MO-allergics	28	31 ± 10	61%	2.7 ± 11.42	Mouse allergic individuals

and stimuli replicates, and a stimulation index ≥2 calculated by performing the ratio of average SFC for stimuli divided by average SFC for negative control. Positive peptide pools were deconvoluted to identify the individual epitopes inducing the response. Supplemental Fig. S1 shows an example picture of ELISPOT data performed on ENA cohort.

Clustering of epitope sequences to account for epitope redundancy

Several reactive peptide epitopes found in this study were amino acid variants of sequences with high homology levels to each other, as such considering them as a single entity would lead to counting the same region multiple times. To correct for redundancy, epitope sequences have been clustered using the cluster-brake option in the cluster 2.0 tool[21] available in IEDB[22] using homology threshold above 70%. Each cluster was counted as a single entity. For each cluster, the epitope yielding the strongest response was selected as representative. Antigen dominance was calculated by summing the response magnitude of the consensus cluster sequences of each antigen.

Ex vivo CD154 assay

Ex vivo T cell responses were measured based on T cell activation assay previously described by Bacher et al.[23] PBMC were thawed and rested overnight, plated at 10×10^6 cells per well in a 6-well plate. The next morning, cells were stimulated with HiMO peptide megapool (2 μg/ml), Phorbol myristate acetate (PMA) and Ionomycin (Io) (positive control) or DMSO (negative control) in the presence of 1 μg/ml anti-CD40 (Miltenyi Biotech, Auburn, CA). Cells were incubated for 6hrs, adding Brefeldin A (1 μg/ml) for the last 3hrs. After the incubation, cells were labeled with anti-CD4 APC ef780, anti-CD3 AF700, anti-CD8/CD14/CD19 V500 and live/dead fixable viability dye (Life technologies, San Diego, CA). After staining and washing, cells were fixed, permeabilized and intracellular staining was performed with anti-CD154 FITC (BD, San Diego, CA). Finally, cells were washed and acquired by flow cytometry using a BD LSR II flow cytometer and data were analyzed using FlowJo software (TreeStar, Ashland, OR). All data acquisition was performed blinded. Gating strategy is shown in Supplemental Fig. S2.

Results

T cell reactivity of ENA against MO antigens

To assay for T cell reactivity in ENA individuals, PBMCs were expanded *in* vitro with urine extracts (3 μg/ml) and cytokine responses (IL-5 and IFNγ) were determined by ELISPOT assay following 24 h restimulation using MO antigen-derived peptides. This approach was previously utilized to define T cell responses in MO-allergic donors.[15] These previously published data are shown replotted here and overall described as "MO-allergics" representing the combinations of rhinitic and asthmatic individuals previously described and used in this study for reference purposes only.[15]

Overall, significant T cells responses were detected in 68% of ENA subjects. As expected, this overall response frequency was lower than that observed in MO-allergics (100% response frequency; $p = 0.0272$ by one-tailed exact Fisher test) (Fig. 1A). In terms of overall response magnitude, a similar picture was noted. The median of the total T cell response/donor in ENA subjects was 547 SFC, compared to 16373 SFC observed in MO-allergics ($p = 0.0004$ by Mann-Whitney test, two-tailed) (Fig. 1B). Analysis of cytokine production revealed that while the T cell response in the allergic cohort is dominated by IL-5, no significant difference is observed between IL-5 and IFNγ production in ENA donors (Fig. .1C and D).

Differences and similarities in T cell targets recognized in ENA versus MO-allergic donors

The data obtained by the epitope screen was further analyzed to establish which antigens are dominantly recognized by ENA T cell responses. The magnitude of responses to be ascribed to each protein was

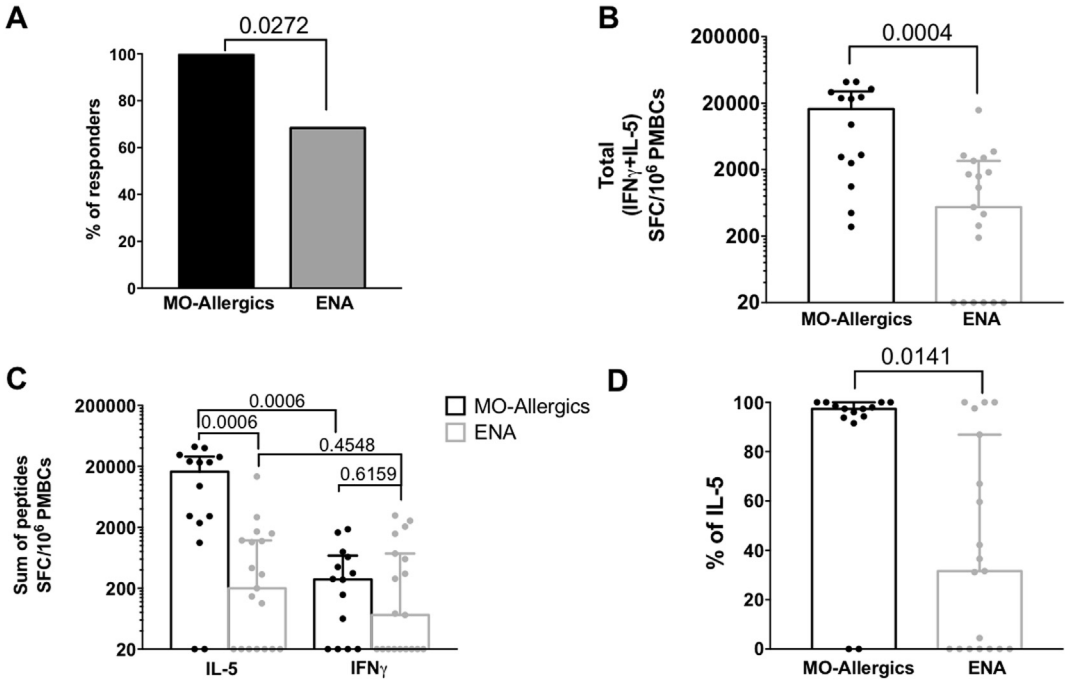

Fig. 1. MO-specific T cell reactivity in ENA (n=19) in comparison with MO-allergics (n=14). A) Percent frequency of responders. B) Response magnitude calculated as the sum of IFNγ and IL-5 responses to the various individual positive peptides C) Cytokine polarization and D) Frequency of IL-5 producing cells out of total response. Data has been generated by IFNγ/IL-5 DUAL ELISPOT after two weeks of *in-vitro* stimulation with HiMO extract. Bar graphs indicate median values, p values are indicated above. Statistical analyses were performed by one-tailed Fisher exact test (panel A) and two-tailed Mann-Whitney test (panel B–D).

calculated as the sum of responses to peptides derived from the respective protein, after redundancy elimination of peptides with ≥70% homology. Antigen dominance in the ENA cohort was compared to data obtained from MO-allergic donors, that was previously published[15] and is again shown replotted here for reference purposes only.

In the ENA cohort, 86.5% of the responses were directed against the family of Major Urinary Proteins (MUPs), which include Mus m 1, Mus m 1 isoforms, and other highly homologous proteins. This pattern of immunodominance is highly similar to that previously observed in MO-allergics, where 87% of the response was directed against these proteins (Fig. 2). More detailed analysis revealed that only kappa-casein precursor and odorant binding protein Ib-like exhibited different immunodominance in the two cohorts. Kappa-casein precursor accounted for 4.3% of total T cell reactivity in MO-allergics but only 0.24% in ENA donors and odorant binding protein Ib-like accounted for 3.4% MO-allergics but only 1.6% in the ENA cohort, respectively (Fig. 2). These data suggest that only minor differences exist at the level of antigenic immunodominance in MO-allergic versus ENA individuals.

Recognition of mouse urinary oligopeptides from ENA individuals

We recently reported[16] that oligopeptides naturally occurring in mouse urine elicit potent T cell responses in MO-allergic individuals. To investigate if these peptides are also targeted by T cell response in the ENA cohort, we assessed ENA T cell reactivity against these oligopeptides, derived from the low molecular weight filtrate fraction of mouse urine (LoMO) described by Antunes et al.,[16] after *in vitro* culture with a megapool containing 225 oligopeptides. After 2-week culture expansion, IL-5 and IFNγ production in response to restimulation with individual peptides was assessed by ELISPOT. The previously published data[16] from MO-allergic donors is shown replotted here for reference purposes only. Overall, significant T cell responses were detected in 90% of ENA subjects, a response frequency similar to that in the MO-allergic cohort (Fig. 3A).

Comparison of the total T cell response magnitude (sum of IL-5 and IFNγ) revealed a non-significant trend for decreased T cell reactivity ($p = 0.096$) in the ENA cohort compared to MO-allergics (Fig. 3B). In terms of cytokine production, both IL-5 and IFNγ were observed in ENA donors, which is similar to what was previously observed in MO-allergics (Fig. 3C and D). These data suggest that small oligopeptides derived from the low molecular weight fraction of mouse urine also elicit T cell responses in ENA individuals, albeit somewhat lower in magnitude compared to MO-allergics.

Detection of MO-epitope specific CD4$^+$ T cells ex vivo responses in ENAs

To further establish the biological relevance of these observations and to exclude that the responses detected were an artifact induced by *in vitro* expansion, we tested a megapool containing the most immunodominant peptides (HiMO megapool) for its capacity to induce *ex vivo* T cell activation in PBMCs from ten MO-allergic and ten ENA donors. Cells were stimulated for 6 h with HiMO megapool followed by staining of the activation marker CD154 (CD40L). Data is expressed as fold change (FC) of CD154 T cell count in stimulated vs unstimulated conditions.

Responses with a stimulation index above 2 to the HiMO pool were seen in 6/10 MO-allergic donors and 3/10 ENA donors (Fig. 4A). Similar results are observed also when analyzing the frequency of CD154 positive cells per million CD4$^+$ cells background subtracted (Fig. 4B). This data confirms the *in vitro* observation reported above, suggesting that mouse-derived T cell epitopes can elicit responses in ENA individuals, with a trend for ENA T cell responses to be less pronounced than those observed in MO-allergic individuals.

Discussion

The data presented herein demonstrate that individuals highly exposed to mouse allergens, yet not sensitized and lacking allergic symptoms do exhibit immune responses to mouse allergens and antigens because of exposure, but the responses are of somewhat different quality and quantity as compared to MO-allergic donors. This finding has been reported in other systems, such as Timothy grass[24] and German cockroach.[25] The present study is the first analysis related to mouse-specific T cell responses in non-allergics, and most importantly studies responses in a population of donors with heavy exposure because of occupational duties. Furthermore, it is the first to analyze T cell responses of exposed donors not only following *in vitro* restimulation, but also directly *ex vivo*, thus eliminating the potential for artifact introduced by the cell culture step.

Analysis of antigen specificity in allergic and ENA donors revealed a high overlap in antigenic dominance, with Mus m 1 and other proteins from the MUP family accounting for over 85% of the total T cell response in both cohorts. Marginal differences were observed for kappa-casein and odorant binding protein 1b, both being a stronger T cell target in MO-allergics compared to ENA donors. This is in contrast with findings in German cockroach allergy,[25] where the T cell response associated with different disease phenotypes was much more heterogeneous. In the cockroach system, we found that T cell responses in asthmatic, rhinitic

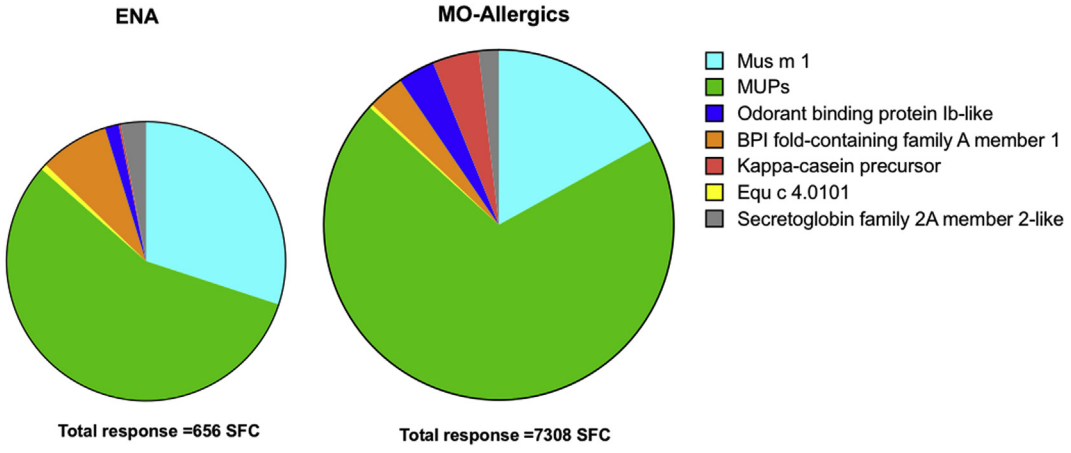

Fig. 2. Antigen immunodominance of ENA (n=19) compared to MO-allergic patients (n=14). Left and right pie charts show antigen immunodominance respectively of ENA and MO-allergics. Responses shown represent the sum of representative epitopes of each antigen after clustering analysis. Data has been generated by IFNγ/IL-5 DUAL ELISPOT after two weeks of *in-vitro* stimulation with HiMO extract. Response of the representative epitopes of each antigen is calculated as the sum of IFNγ and IL-5 after using cluster-break method implemented in cluster 2.0 available in IEDB[21] (http://tools.iedb.org/main/).

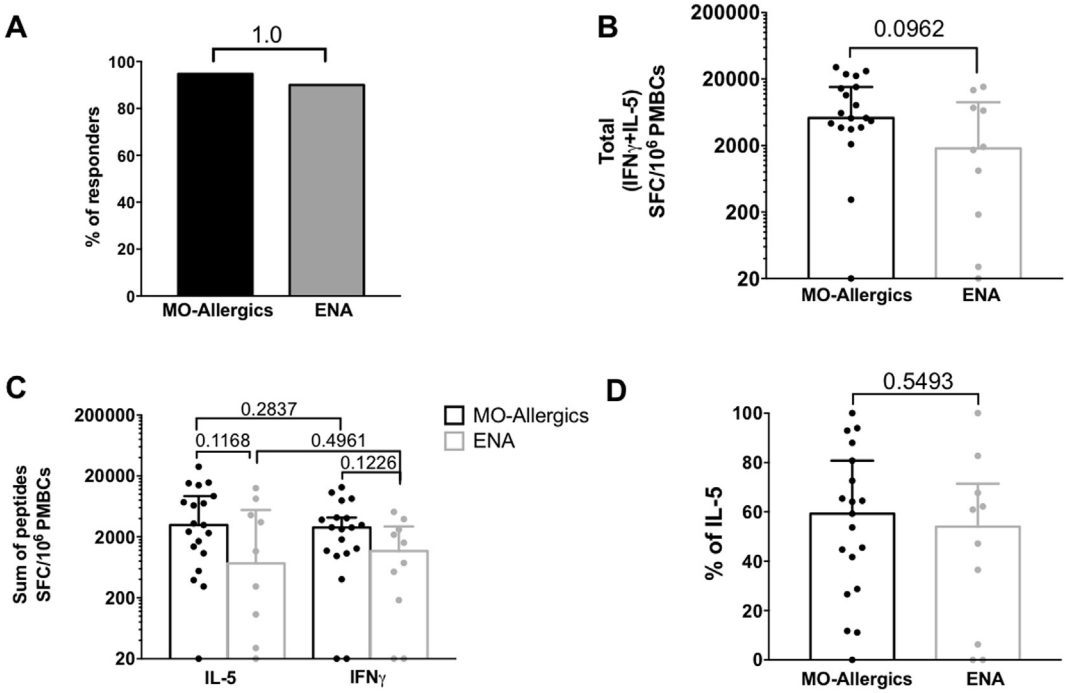

Fig. 3. MO-specific T cell reactivity against mouse urinary oligopeptides. MO-allergics (n = 19) are shown in black, ENA (n = 10) in grey. A) Frequency of responders. B) Response magnitude calculated as the sum of IFNγ and IL-5 responses C) Cytokine polarization and D) Percent of IL-5 producing cells out of total response. Data has been generated by IFNγ/IL-5 DUAL ELISPOT after two weeks of *in-vitro* stimulation with Low Mo pool of peptide. Statistical analysis was performed by Fisher exact test (panel A) and Mann-Whitney test, two-tailed (panel B–D). Bars indicate median, p values are shown above.

and non-allergic donors were associated with distinct immunodominance patterns of different antigens. This difference may be related to the strong immunodominance of major urinary proteins observed in mouse allergy compared to German cockroach, where 10 different allergens are currently reported in the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database,[26] while Mus m 1 is the only known allergen listed for mouse allergy in the WHO/IUIS database.

In terms of overall T cell response magnitude, our study shows that, as expected, responses were lower in the ENA cohort as compared to MO-allergic donors. However, strikingly, these differences did not always reach statistical significance, highlighting that non-sensitized individuals do not remain oblivious to environmental allergens to which they are exposed, as also reported in the Timothy grass[24] and German cockroach[25] systems, where allergen-specific T cell responses in non-allergic individuals were frequently detected. Furthermore, in studies that compared overall magnitude of T cell responses between different levels of disease severity, rather marginal differences were seen for example between severe vs mild to moderate asthma[24] or even asthmatic and rhinitic donors,[15,25] suggesting that overall T cell response magnitude is not a strong correlate of disease or allergic symptom severity. Functionally, we did observe a significant difference in cytokine polarization for MO-allergics, which were Th2-polarized, compared to ENA donors, which exhibited similar levels of IL-5 and IFNγ production in response to

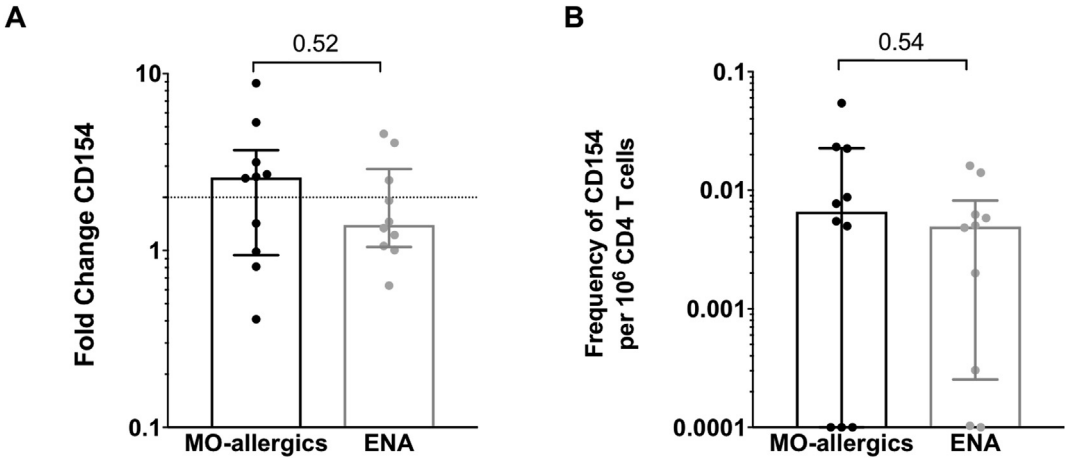

Fig. 4. Differences in *ex vivo* T cell activation (CD154 expression) in MO-allergics (n=10) versus ENA donors (n=10). A) CD154 fold change expression and B) CD154 frequency per million $CD4^+$ T cells over background is shown. Cells were stimulated for 6 h with HiMO megapool or DMSO as negative control, membrane staining was performed followed by CD154 + ICS. Gating strategy is shown in Supplementary Fig. S2, the number of CD154 + cells was calculated per million $CD4^+$ and plotted as fold change or as frequency of response background subtracted. Statistics were performed using Mann-Whitney test, two-tailed.

HiMO peptides. Interestingly, no difference in cytokine polarization was detected between MO-allergics and ENA subjects in response to the oligopeptides derived from the low-molecular weight fraction. It is possible that the T cell epitopes most dominantly targeted by the Th2 response of these MO-allergic donors were under-represented in the low molecular weight fraction compared to the HiMO peptides tested, leading to a small shift in the ratio of IL-5 and IFNγ.

The molecular pathways associated with the T cell responses of allergic and non–allergic donors warrant further investigation. Our lab has previously reported that exposure to pollen allergens is associated with specific transcriptomic changes[24] in non-allergic donors. Comparison of the gene expression profiles in versus out of pollen season in antigen-specific cells from non-allergic subjects revealed a signature consistent with a downmodulation of IL-5 producing cells.[4] Moreover Ahuja et al.[27] reported characteristic changes at the level of RNA expression profiles and skin barrier function upon HDM allergen exposure in non-allergic donors. In future experiments, we plan to further address these issues by detailing potential transcriptomic differences between MO-exposed allergic and non-allergic donors. Moreover, we plan to assess the quality of the response in non-allergic subjects in terms of other cytokines such as IL-9, IL-22, IL-17A and the extend of IL-10, TGFβ and Fox p 3 expression in CD154 + cells.

Declarations

Ethics approval and consent to participate

All study participants were recruited from San Diego, CA, following Institutional Review Board approval by the La Jolla Institute's Institutional review board (La Jolla Institute for Allergy and Immunology, La Jolla, CA, IRB protocol no. 112–0217). All patients enrolled in this study provided written consent.

Consent for publication

All contributing authors consent to this publication.

Availability of data and material

All data and material are presented in this manuscript. Raw data can be made available upon request.

Competing interests

The author do declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2019.100026.

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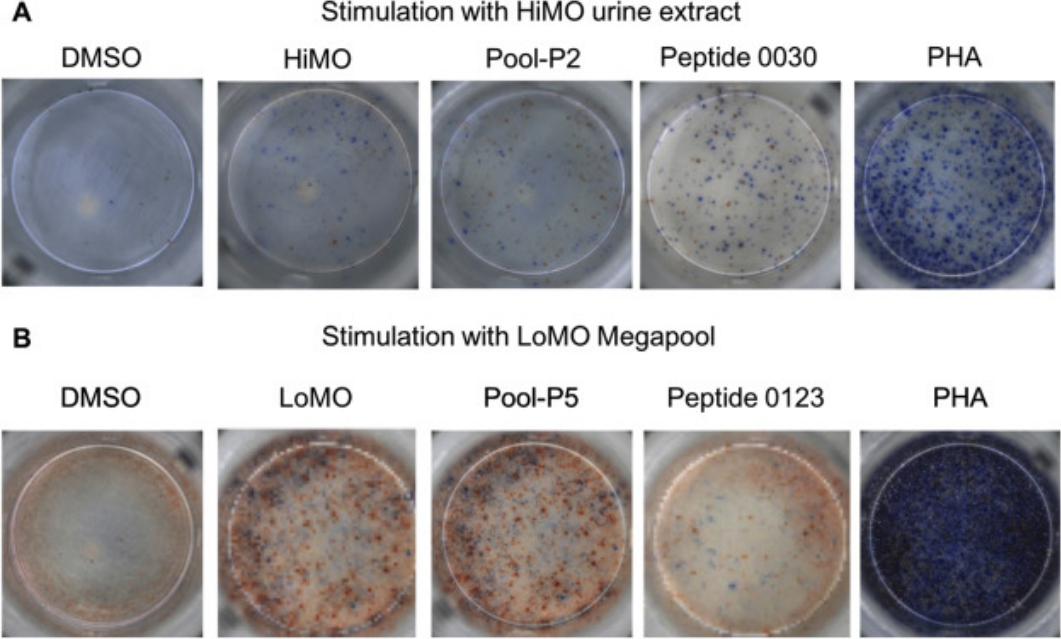
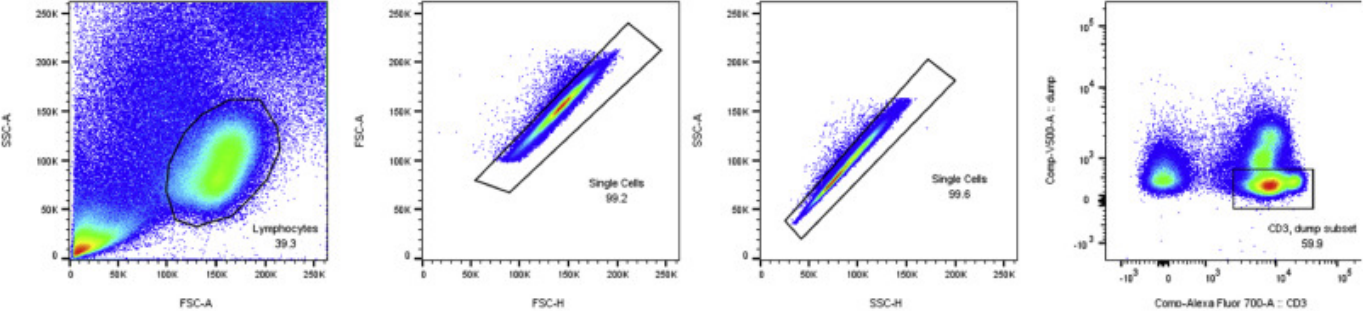
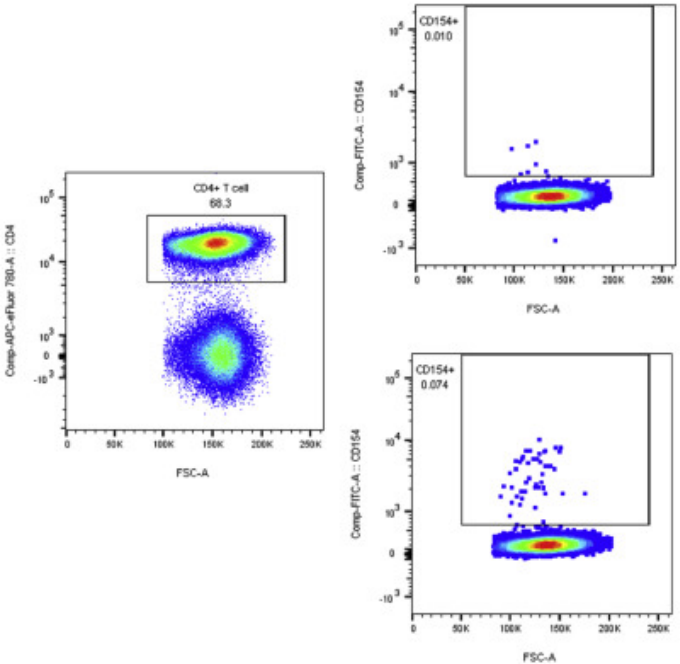

A
Stimulation with HiMO urine extract
DMSO
HiMO
Pool-P2
Peptide 0030
PHA
B
Stimulation with LoMO Megapool
DMSO
LoMO
Pool-P5
Peptide 0123
PHA





Medium with DMSO

HiMO peptide pool