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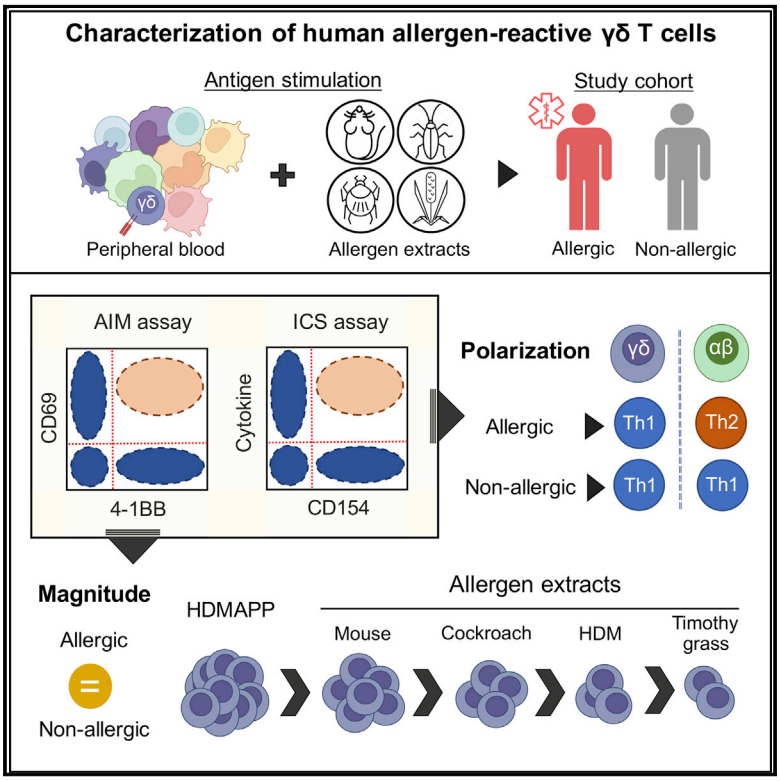
Ex vivo assays show human gamma-delta T cells specific for common allergens are Th1-polarized in allergic donors

Graphical abstract



Highlights

- *Ex vivo* assays using peripheral blood can detect human γδ T cell responses
- Upregulated 4-1BB and CD69 expression tag allergen-reactive human γδ T cells
- γδ T cell responses are donor and allergen specific and abrogated by TCR blocking
- Mouse and cockroach allergen-specific γδ T cell responses are Th1-polarized

Authors

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In brief

Yu et al. characterize human gamma-delta (γδ) T cells in peripheral blood using *ex vivo* assays. They find that human γδ T cells specific for common environmental allergens are Th1-polarized in allergic donors. γδ T cells could be of immunotherapeutic use to rebalance dysregulated Th2 responses in allergic diseases.

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Ex vivo assays show human gamma-delta T cells specific for common allergens are Th1-polarized in allergic donors

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MOTIVATION Current methodologies for studying gamma-delta (γδ) T cells are limited to clonal expansion and long-term *in vitro* cultures. As a result, insufficient insights are offered to fully understand the role of γδ T cells in human diseases. Here, we report the development of a methodology that reliably identifies antigen-reactive γδ T cells in human peripheral blood mononuclear cells (PBMCs) both quantitatively and qualitatively. A characterization of antigen-reactive γδ T cells was performed for several allergen extracts in allergic and non-allergic individuals. However, no major differences were observed, likely due to the complex composition of allergen extracts, which requires further studies on individual antigen components.

SUMMARY

Gamma-delta (γδ) T cells contribute to the pathology of many immune-related diseases; however, no *ex vivo* assays to study their activities are currently available. Here, we established a methodology to characterize human allergen-reactive γδ T cells in peripheral blood using an activation-induced marker assay targeting upregulated 4-1BB and CD69 expression. Broad and reproducible *ex vivo* allergen-reactive γδ T cell responses were detected in donors sensitized to mouse, cockroach, house dust mite, and timothy grass, but the response did not differ from that in non-allergic participants. The reactivity to 4 different allergen extracts was readily detected in 54.2%–100% of allergic subjects in a donor- and allergen-specific pattern and was abrogated by T cell receptor (TCR) blocking. Analysis of CD40L upregulation and intracellular cytokine staining revealed a T helper type 1 (Th1)-polarized response against mouse and cockroach extract stimulation. These results support the existence of allergen-reactive γδ T cells and their potential use in rebalancing dysregulated Th2 responses in allergic diseases.

INTRODUCTION

Gamma-delta (γδ) T cells are a relatively small subset of "unconventional" T cells in peripheral blood but are one of the primary T cell subsets in mucosal epithelia where encounters to allergens happen.[1] γδ T cells are also endowed with versatile and pro-inflammatory activities[2–5] and are known to contribute to the pathology of many human immune-related diseases induced by infection, autoimmunity, or cancer.[5–9] Recent findings suggest a potential role for γδ T cells in food allergy, allergic asthma, or in mediating allergic airway inflammation both by regulating immunoglobulin E (IgE) production in B cells or by direct cell contact and cytokine production.[10–15] It has been reported that γδ T cells might promote airway inflammation in response to cockroach (CR) challenge[16] and in the modulation of house dust mite (HDM) allergen-specific T-helper type 2-skewed immunity.[17] γδ T cells derived from nasal mucosa in allergic subjects have also been shown to recognize pollen extracts and drive IgE production both *in vitro* and *in vivo*[18] and promote exacerbation of allergic conjunctivitis.[19] Conversely, resolution of allergic airway hyperactivity after long-term allergen challenge required active suppression from γδ T cells.[20] It is important to point out that the majority of this research has been performed in mouse challenge models or human observational studies with no or few

mechanistic insights.[21] Therefore, it is important to investigate whether allergen-reactive γδ T cells exist in humans and effectively play a role in allergic reactions. However, the lack of experimental assays to identify and characterize human allergen-reactive γδ T cells has hampered progress.

γδ T cells defined by the expression of the heterodimeric T cell receptors (TCRs), composed of γ and δ chains, fall into two major subtypes, Vδ1 and Vδ2 T cells, which vary in TCR-γ chains and function at distinct anatomic locations.[4,22,23] Vδ1 T cells are primarily tissue specific and reside mainly in the epithelium (skin) or in mucosal-rich tissues (e.g., intestines, lungs, etc.), while Vδ2 T cells, which can be recruited to inflamed tissue, account for the majority (75%–95%) of circulating γδ T cells in the blood.[23,24] Importantly, γδ T cells can display broad functional activities with secretion of chemokines and cytokines, in particular with pro-inflammatory activities such as interferon (IFN)γ, interleukin-17 (IL-17), and tumor necrosis factor α (TNF-α).[24,25] γδ T cells can also express innate immune receptors such as Toll-like receptors (TLRs) and natural killer receptors (NKRs), leading to the regulation of cytotoxicity and release of cytolysis granules (e.g., perforin and granzymes).[4,26] These γδ T cell effector functions are of growing interest for their potential use in cancer immunotherapy, autoimmune disorders, and infectious or allergic diseases.[21,25,27]

γδ T cells have been reported to recognize a wide variety of antigens and non-polymorphic ligands.[26] Interestingly, the expression of γδ T cell ligands is modulated by the particular physiological context in the blood or tissue[28,29] and diverges significantly between humans and mice,[30] further illustrating the need to deepen our knowledge of human γδ T cells. To date, the most powerful ligands used to stimulate human γδ T cells are phosphorylated metabolites such as microbial (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HDMAPP) or eukaryotic isoprenoid precursor isopentenyl pyrophosphate (IPP).[31] These compounds are routinely used for differentiation or selective (clonal) expansion of γδ T cells from peripheral blood mononuclear cells (PBMCs) after long-term *in vitro* cultures.[32–34] Currently, methodologies to identify and characterize antigen-reactive γδ T cells *ex vivo* are scarce[35] or non-existent in the context of allergic diseases.

Here, we developed *ex vivo* activation-induced marker (AIM) and intracellular cytokine staining (ICS) assays to detect allergen-specific γδ T cells in human PBMCs stimulated with allergen extracts. We characterized their phenotypes and functional responses in response to mouse (MO), CR, HDM, and timothy grass (TG) allergens.

RESULTS

Method development: Direct detection of human γδ T cell responses by an AIM assay

Previous studies that established methodologies to expand and characterize γδ T cells rely mainly on long-term *in vitro* cultures using phosphoantigens or other strong "activator" compounds.[32–34] However, assay methodologies to characterize human antigen-specific γδ T cell responses directly *ex vivo* have not yet been described. As a result, scarce data are available to dissect the potential involvement of γδ T cells in allergic diseases. Here, we explored whether an AIM assay, similar to the one commonly used to detect conventional antigen-specific cells αβ T cells,[36,37] could be applied to detect human reactive γδ T cell responses. Specifically, we measured the upregulation of the activation markers 4-1BB (CD137) and CD69 in human PBMCs. These two markers have been used in AIM assays for $CD4^+$ and $CD8^+$ T cells, and we expected them to be suitable for γδ T cells as well since 4-1BB signaling is associated with activation, expansion, and effector functions of γδ T cells in mice and humans,[38] while CD69, a classical early marker of activation, is a tissue retention marker for human γδ T cells.[39]

Utilizing this assay modality, strong responses were detected against HDMAPP, the most potent known activator of γδ T cells, which is commonly used for expanding γδ T cells *in vitro*[31] Specifically, all donors tested (48/48) were responsive with a median magnitude of 82.7% (8.06%–94%) reactive γδ T cells, as shown by the upregulation and dual expression of CD137 and CD69. As expected, no response to HDMAPP stimulation was observed when conventional αβ T cells were analyzed (Figures S1A and S1B). Only a minimal response was observed when PBMCs were left unstimulated or cultured with media (negative control). These residual values (background activation levels) were subtracted from all data for each HDMAPP or stimuli-specific responses throughout the study (also, see method details for criteria of positivity). The reproducibility of this assay was demonstrated by plotting γδ T cell responses of 34 donors to HDMAPP stimulation in multiple independent experiments conducted on different days and by calculating the coefficient of variation (CV = 0.08), which further confirmed the data's low variability in multiple replicates (Figure S1C).

Direct *ex vivo* detection of allergen-reactive human γδ T cells for several common allergens

Next, we explored whether allergen-specific γδ T cell reactivity could be detected in PBMCs from allergic donors to four common allergens: MO, CR, HDM, and TG. PBMCs from a cohort of 153 donors sensitized for the different allergens as defined by allergen-specific IgE titers of >0.35 kUA/L were utilized. Detailed clinical and demographic data are described in Table S1.

Representative data for γδ T cell reactivity using the AIM assay and stimulation with a MO allergen extract, which we have previously extensively characterized in the study of conventional mouse-specific αβ T cells responses,[40–43] is shown in Figure 1A. MO-specific γδ T cell responses were readily observed in all donors (33/33) with a wide range of reactivity (median: 16.91%, range: 2.07%–50.42%) (Figure 1B). Levels of background activation seen without stimulation (Neg) are measurable and also differ between individuals, albeit approximately (approx.) 95-fold lower in magnitude than MO-specific responses (median: 0.179%, range: 0.028%–1.53%). Similar to the positive control (HDMAPP), the vast majority of MO-allergen-stimulated γδ T cells were from the Vδ2 subset (Figure 1C). Responding cells were predominantly $CD4^-CD8^-$ (double-negative cells) (Figure S2). This phenotypic profile resembles the one associated with the majority of circulating γδ T cells in human blood.[23,24]

We also measured the response from conventional αβ T cells to the same MO allergen as previously described.[36,41] Strikingly,

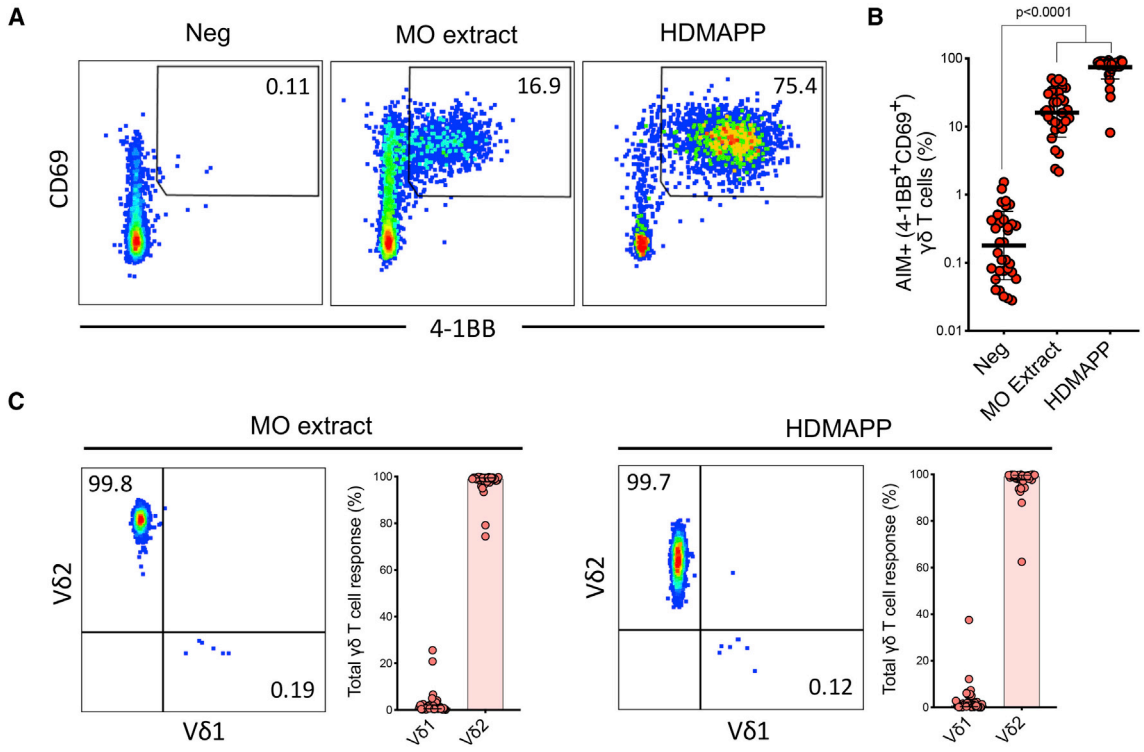

Figure 1. γδ T cell reactivity to a mouse allergen extract can be detected using an AIM assay

Allergen-specific γδ T cell responses were measured as percentage of AIM$^+$ (CD137$^+$CD69$^+$) γδ T cells after stimulation of PBMCs with MO extract, HDMAPP, or media (Neg) as control.

(A) Representative fluorescence-activated cell sorting (FACS) plot of each condition.

(B) Percentages of allergen-specific γδ T cells responding to the indicated stimulations across all donors.

(C) (Left) Representative FACS plots of the two γδ T cell subsets (Vδ1 versus Vδ2) gated on MO or HDMAPP AIM$^+$ γδ T cells. (Right) Bars show percentage of each subset across all donors.

All graphs show data represented as geometric mean with SD. Each dot represents a unique individual (MO, n = 33; HDMAPP, n = 48). Pairwise comparisons were performed with the Wilcoxon test, and p values <0.05 were considered statistically significant.

γδ T cell reactivity to MO allergen extract accounted for the majority of the CD3$^+$ T cell response (Figures 2A and 2B). In the case of stimulation with a pool of previously described MO-allergen-derived peptides,[41] αβ T cell response was detected but γδ T cell reactivity was not, suggesting that in MO allergen extract a non-peptidic antigen may be recognized by γδ T cells (Figures 2C and 2D). Alternatively, it is possible that αβ and γδ T cells may recognize different peptide antigens.

We extended our findings to other common allergens (CR, HDM, TG) and observed γδ T cell responses in multiple allergic donors (Figures 3A and S2). Overall, 95.8%, 85.4%, and 54.2% of the donors were positive for CR, HDM, and TG, respectively (see method details for criteria of positivity); the median magnitudes of γδ T cell response were 1.15% (0.01%–14.35%), 0.27%, (0.01%–6%), and 0.02% (0.01%–0.24%) for CR, HDM, and TG, respectively. As opposed to the αβ T cell reactivity (Figure 3B), the magnitude of γδ T cell responses varied among the different allergen extracts, with the highest reactivity against MO extract, followed by CR, HDM, and TG (Figure 3A). Similar results were observed when plotting the same data as a function of stimulation index (SI) (Figures S3A and S3B). Therefore, the fraction of total extract response accounted by γδ T cells versus αβ T cells varied in an allergen-specific fashion (Figure S3C). While reactivity to MO extract is largely due to the γδ T cell response, similar to HDMAPP (Figure S1), comparable levels of reactivity were observed for αβ and γδ T cells in response to CR extract. In contrast, for the HDM and TG extracts, the largest fraction of response was associated with αβ T cells (Figure S3C). These results further suggest that reactivity to different extracts is allergen specific and that each extract might contain different compounds responsible for γδ T cell antigenicity.

In conclusion, human γδ T cells reactive to 4 different allergen extracts can be readily detected *ex vivo* in 54.2%–100% of allergic subjects tested and comprised 0.02%–16.91% of a specific activation in the total γδ T cell population. The donor- and allergen-specific pattern of reactivity suggests that different compounds elicit γδ T responses in different subjects.

Differential allergen-specific mechanisms of γδ T cell activation by allergen extracts

Different allergen extracts, owing to their complexity,[44] might contain different immunogenic compounds recognized by γδ T cells. Indeed, in the case of MO allergen extract, the γδ T cell responses are elicited by the low molecular weight fraction of the

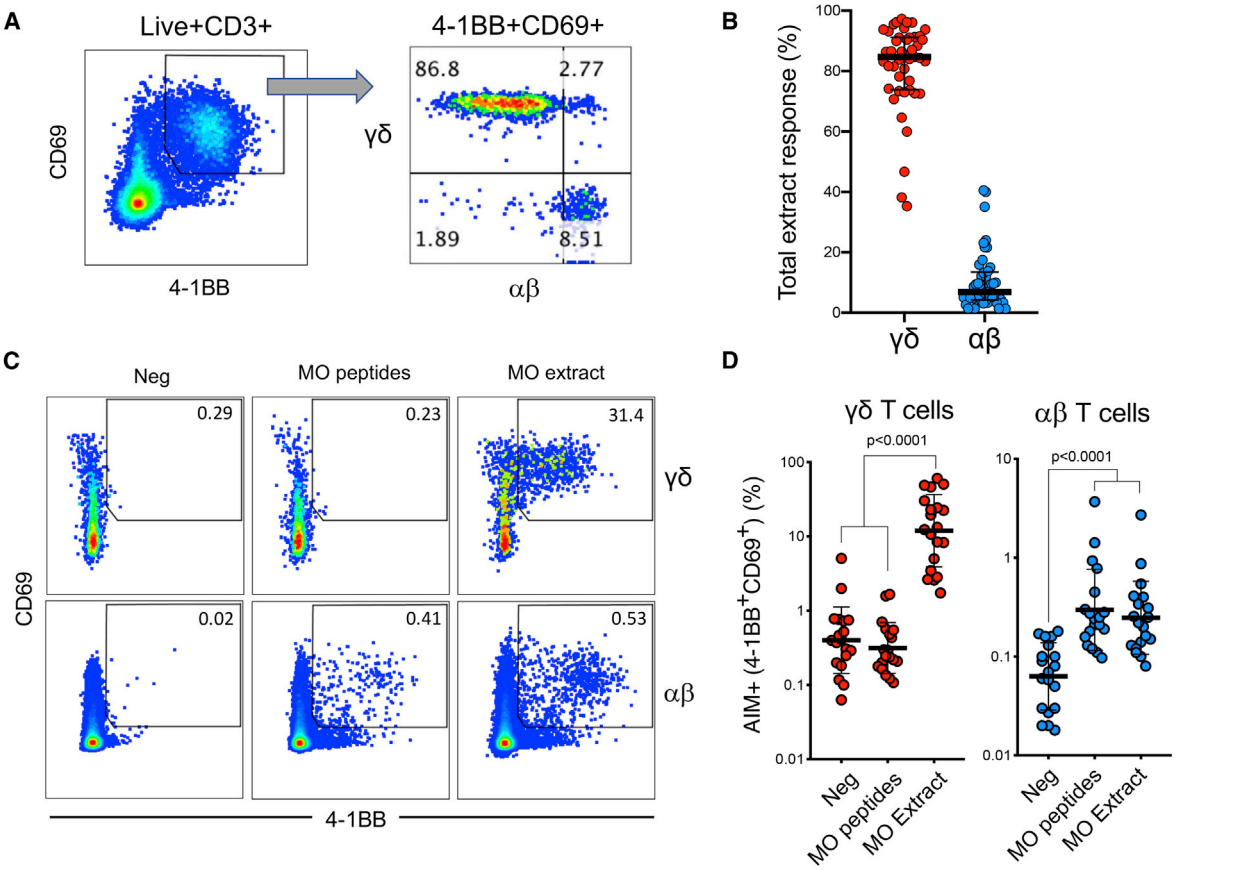

Figure 2. γδ T cell reactivity accounts for the majority of the mouse extract response and is not mediated by peptides

Total MO-specific T cell responses were measured as a percentage of AIM$^+$ (CD137$^+$CD69$^+$) CD3$^+$ T cells after stimulation of PBMCs with MO extract, and the individual γδ and αβ T cell reactivity was assessed.

(A) Representative FACS plot of total AIM$^+$CD3$^+$ T cells (left) further gated as function of γδ and αβ T cell reactivity (right)

(B) Graph shows the relative percentage of γδ and αβ reactivity from the total MO-specific T cell responses across all donors (n = 33).

(C) (Left) Representative FACS plots of AIM$^+$ (CD137$^+$CD69$^+$) γδ (top row) or αβ (bottom row) T cells after stimulation of PBMCs with MO peptide pools (MPs), MO extract, or control (Neg; media for γδ T cells, or DMSO for αβ T cells).

(D) Graphs show the percentage of AIM$^+$ reactivity in each condition for γδ or αβ T cells across all donors (n = 20).

All graphs show data represented as geometric mean with SD. Each dot represents a unique individual. Pairwise comparisons were performed with the Wilcoxon test, and p values <0.05 were considered statistically significant.

extract (i.e., <3 kDa), while the antigenic fraction in CR allergen extract is of high molecular weight (i.e., >3 kDa) (Figures S4A–S4C). This observation led to the hypothesis that a different mechanism of action might also mediate the activities of MO- and CR-specific γδ T cell responses. To determine whether the activation of γδ T cells by allergen extracts is T cell-directed or mediated by other cell types present in PBMCs, antigen-presenting cells (APCs) were depleted from the PBMC preparations (Figure 4). The effect of MO allergen extract stimulation in γδ T cells is not negatively impacted by the absence of APCs (Figure 4A). Conversely, the activation of γδ T cells by CR allergen extract is completely abrogated by the absence of APCs (Figure 4B), while HDMAPP stimulation shows only a slight reduction (Figure 4C). These results should be interpreted with caution given the experiment's low group size.

To address if γδ T cell activation in response to allergen extracts is elicited through TCR signaling pathways and not innate signaling pathways, PBMCs were cultured in the presence of dasatinib, a known TCR blocking reagent.[45] γδ T cell responses to MO or CR allergen stimulation as well as to HDMAPP or α-CD3, a positive control, were completely abrogated when PBMCs were cultured with dasatinib (Figure 4D). The same results were observed when analyzing responses elicited by conventional αβ T cells (Figure 4E). As expected, lipopolysaccharide (LPS) stimulation accounted only for a small fraction of the γδ T cell reactivity and was not affected by the use of dasatinib (Figures S4D and S4E). In conclusion, the data shown herein suggest that human γδ T cell reactivity to allergen extracts is potentially associated with different allergenic antigens and mediated by mechanisms involving TCR signaling. While the role of APCs in mediating γδ T cell activation might differ between allergen extracts, the contribution of αβ T cells needs to be determined.

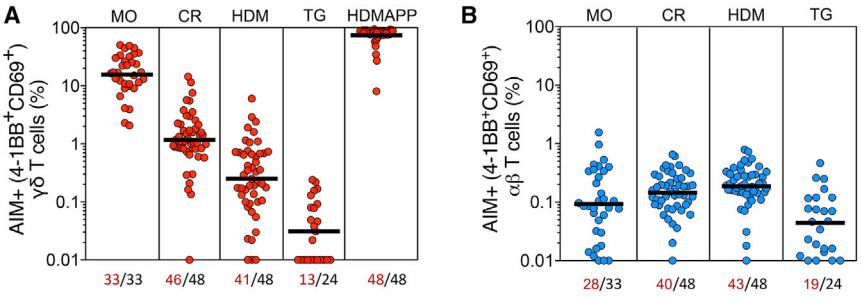

Figure 3. γδ and αβ T cell reactivity is detected with MO, CR, HDM, and TG extracts

(A and B) Allergen-specific T cell responses were measured as percentage of AIM$^+$ (CD137$^+$CD69$^+$) cells after stimulation of PBMCs with mouse (MO), cockroach (CR), house dust mite (HDM), and timothy grass (TG) extracts or HDMAPP. Graphs show percentages of allergen-specific (A) γδ T cells or (B) αβ T cells responding to the indicated stimulations across all donors (MO, n = 33; CR, n = 48; HDM, n = 48; TG, n = 24). Each dot represents a unique individual, and geometric mean and number of donors associated with a positive response (red) for each stimulus is shown.

Allergen-reactive human γδ T cell responses are detected in non-allergic subjects

Previous studies established that allergen-specific αβ T cell responses are also readily detected in non-allergic subjects, albeit with different frequencies and/or functionality when compared with allergic subjects.[40,42,43,46,47] Accordingly, we next examined whether allergen-specific γδ T cell responses could also be detected in non-allergic donors. To this end, we utilized PBMCs from a cohort of 39 healthy donors who had no clinical symptoms for MO, CR, HDM, or TG allergies and negative laboratory evidence of sensitization as defined by plasma-allergen-specific IgE levels of <0.10 kUA/L. Demographic and clinical information are summarized in Table S2.

The data in Figure 5 depict the magnitude of γδ T cell responses to the various extracts in non-allergic donors (gray) compared with allergic donors (red). Allergen-extract-specific γδ T cell responses were readily observed in non-allergic donors with frequencies comparable to those observed in allergic donors (38.5%–100%) (Figure 5). The magnitude of MO-, CR-, or TG-specific γδ T cell responses was indistinguishable between non-allergic and allergic donors, while the magnitude of γδ T cell reactivity in response to HDM had a trend for lower reactivity ($p = 0.047$) in non-allergic donors (Figure 5). Lowered numbers of circulating γδ T cells have been described in the context of allergies.[48] To account for potential differences in the proportion of responding γδ T cells as part of all circulating lymphocytes, responding γδ T cells were plotted as a percentage of total CD3$^+$ T cells and the magnitude compared between non-allergic and allergic cohorts (Figure S5). Similar to data in Figure 5, this analysis further confirmed that there are no significant observable differences in the magnitude of γδ T cell responses between sensitized versus non-sensitized cohorts.

Finally, γδ T cell reactivity to the allergen extracts did not differ in Vδ1/Vδ2 subsets or CD8/CD4 expression between non-allergic and allergic donors (data not shown). In conclusion, γδ T cell responses are also readily detected in non-allergic subjects, like has been previously described for allergen-specific αβ T cell responses.

The γδ T cells reactive to MO and CR allergens are Th1-polarized

Conventional αβ T cell responses to allergen extracts are associated with a differential T helper type 2 (Th2)/Th1 polarization phenotype in allergic versus non-allergic individuals.[40–42,47] Here, we evaluated cytokine responses by ICS of T cells responding to allergen-specific stimulation as measured by CD154 (CD40L) activation marker expression, as previously described.[40–42,49] PBMCs from a cohort of 12 donors sensitized for both MO and CR allergens, as defined by allergen-specific IgE titers of >0.35 kUA/L, were utilized. Twelve non-allergic donors were used as controls. Detailed clinical and demographic data are described in Table S3.

More specifically, IFNγ, TNF-α, IL-4, and IL-10 secretion was measured (Figure 6). The HDMAPP control was associated with Th1-polarized responses (IFNγ and TNF-α cytokine production) in both allergic and non-allergic donors (Figure 6A). Representative data for each cytokine are shown in Figure S6A. The γδ T cells reactive to MO extract were also strongly Th1-polarized in both allergic donors and non-allergic donors (Figure 6B). Reactivity of γδ T cells to the CR extract also exhibited a Th1-polarized profile with significantly higher magnitude in allergic donors compared with non-allergic donors ($p = 0.005$ and 0.018 for IFNγ and TNF-α, respectively) (Figure 6C). In addition, for each multi-sensitized donor, MO and CR total cytokine responses were compared against each other, revealing that certain subjects could be relatively high responders to one allergen extract and also low responders for the other allergen extract (Figure S6B). This lack of correlation indicates canonical antigen specificity, which differs in each allergen/donor combination.

In summary, we found that γδ T cell reactivity against allergen extracts is mostly associated with a Th1-polarized profile in allergic donors, which is in stark contrast to the Th2 profile of αβ T cells, a hallmark of allergic responses.[40,50,51]

DISCUSSION

Detection of antigen-specific γδ T cells and the study of their role in the context of human pathologies is a major challenge, in part due to the paucity of immunological tools to characterize this T cell subset *ex vivo*.[52,53] Herein, we report the development of an AIM assay that allows detection and characterization of human allergen-specific γδ T cells.

Strikingly, we found that γδ T cells specific for common allergens are readily detected, abundant, and Th1-polarized in allergic donors but are also present in non-allergic individuals. To the best of our knowledge, this is the first report of *ex vivo* detection and functional characterization of human

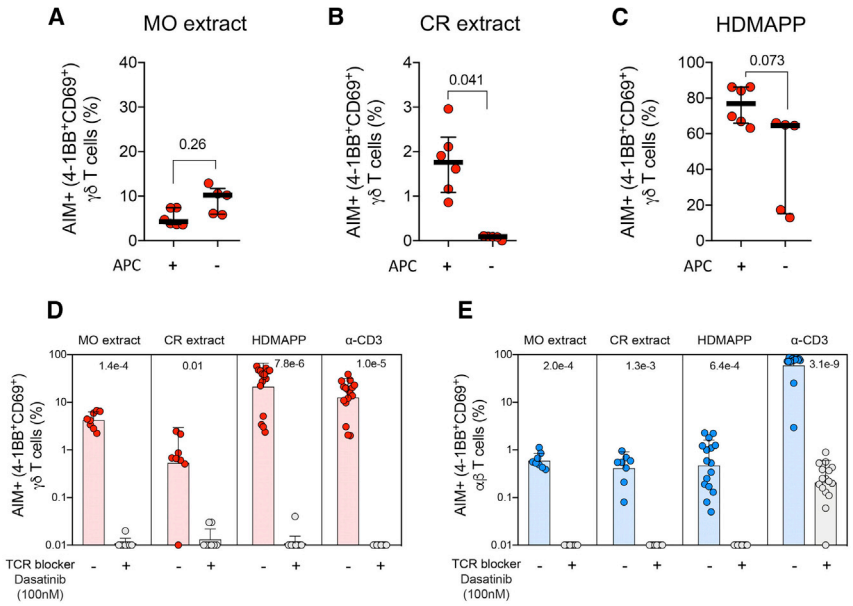

Figure 4. γδ T cell reactivity to MO and CR allergen extracts is TCR specific, albeit with different requirements for the presence of APCs

(A–C) Allergen-specific γδ T cell responses were measured as percentage of AIM$^+$ (CD137$^+$CD69$^+$) γδ T cells after stimulation of PBMCs with (A) MO extract, (B) CR extract, or (C) HDMAPP in the presence (+) or absence (–) of antigen-presenting cells (APCs).

(D and E) PBMCs were stimulated with an MO or CR extract, HDMAPP, or α-CD3 and cultured in the absence (–) or presence (+) of a TCR blocking reagent (dasatinib). Graphs show percentage of AIM$^+$ γδ (D) or αβ (E) T cells in response to the different stimuli and conditions (MO, n = 8; CR, n = 8; HDM, n = 16; TG, n = 16).

Each dot represents a unique individual. Data are represented as geometric mean and SD. Kruskal-Wallis or Wilcoxon paired tests were performed, and p values are indicated. $p < 0.05$ was considered statistically significant.

antigen-specific γδ T cells. This is of importance as current methodologies employed in functional studies of γδ T cells rely exclusively on long-term *in vitro* expansion and physiological manipulation.[53–55]

We studied γδ T cell-specific-responses against four common allergens using MO, CR, HDM, or TG allergen extracts obtained from natural allergen sources. After stimulation, allergen-specific γδ T cells were observed for all extracts and with a large dynamic range among different extracts. Interestingly, for the allergen extracts exhibiting the most potent responses, γδ T cell reactivity was equal (CR) or even higher (MO) than the reactivity from conventional αβ T cells. These are novel observations that have been previously underappreciated and could influence the result of allergen immunotherapies (AITs) that use different allergens or different batches of allergen extracts, especially given the fact that CR and MO extracts are currently non-standardized.[56,57] We predict that the knowledge gained from these findings could have important implications in AIT strategy design and result in a better understanding of the clinical outcomes following treatment initiation.

Allergen-reactive γδ T cell responses were associated with Th1-type functionality, and the cytokine secretion profile of γδ T cells in response to MO and CR extracts in allergic donors was dominated by IFNγ and TNF-α. These results were not unexpected since γδ T cells are, in general, associated with a Th1 profile.[2,4] However, this is in stark contrast to the Th2-polarized pattern of αβ T cells in allergic donors, a general hallmark of allergic responses,[50,58–60] and those previously observed in response to MO and CR allergen extracts.[40,41,46] We hypothesize that antigens that activate γδ T cells and are contained in the allergen extracts might provide a safe avenue to modulate allergen responses by stimulating a Th1 response and rebalance or skew deregulated Th2 responses in allergic diseases. Our study indicates that a similar experimental approach could be applied to study the potential role of γδ T cells in other human pathologies, as dysregulated γδ T cells appear to be a common

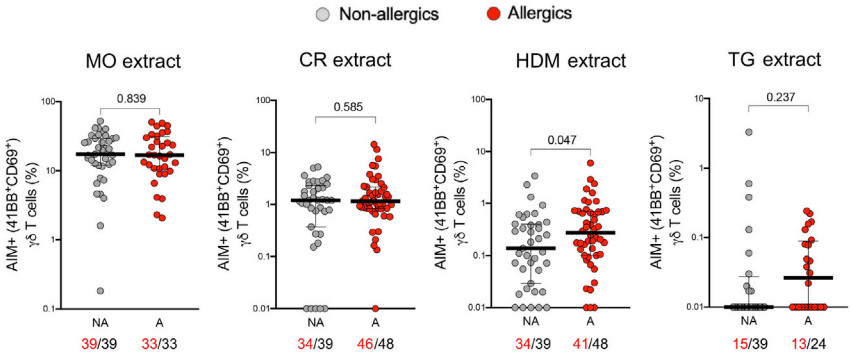

Figure 5. Magnitude of allergen-reactive γδ T cells is similar between allergic and non-allergic donors

Allergen-specific T cell responses were measured as percentage of AIM$^+$ (CD137$^+$CD69$^+$) γδ T cells after stimulation of PBMCs with MO, CR, HDM, or TG extract. Graphs show percentages of allergen-specific γδ T cells in non-allergic (gray) or allergic donors (red) responding to the indicated stimulations across all donors (allergics: MO, n = 33; CR, n = 48; HDM, n = 48; TG, n = 24; non-allergics: n = 39).

Each dot represents a unique individual, and geometric mean is shown. Number of donors associated with a positive response (red) for each stimulus is indicated in the bottom. Mann-Whitney test was performed, and p values are indicated. $p < 0.05$ was considered statistically significant.

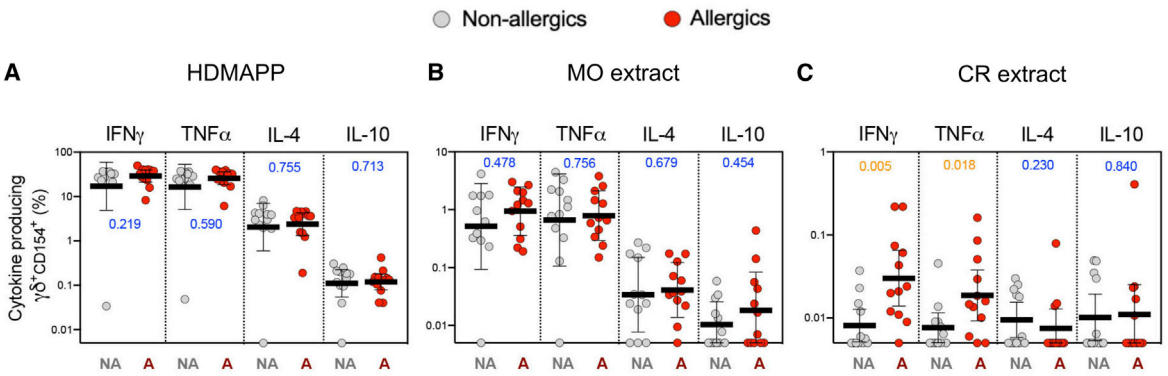

Figure 6. MO and CR extracts elicit Th1-polarized cytokines from γδ T cells in allergic donors

γδ T cell cytokine responses (IFNγ, TNF-α, IL-4, and IL-10) were measured by intracellular cytokine staining (ICS) of γδ T cells responding to allergen-specific stimulation as measured by CD154 (CD40L) expression. Graphs show percentage of AIM$^+$ (CD154$^+$cytokine$^+$) γδ T cells for non-allergic (gray) or allergic donors (red) responding to (A) HDMAPP, (B) MO, or (C) CR extracts (allergics: n = 10; non-allergics: n = 10). Each dot represents a unique individual, and geometric mean for each stimulus/cytokine is shown. Mann-Whitney test was performed, and p values are indicated. $p < 0.05$ was considered statistically significant.

feature of persistent or chronic infectious and inflammatory conditions as well as cancers.[1,61] Future studies could also focus on the possible advantages of combining current γδ T cell-based immunotherapies, which have shown limited efficacy after γδ T cell *ex vivo* expansion,[62] with allergens that are potent pro-inflammatory inducers and well tolerated by non-allergic donors.[63–65]

Our data suggest that γδ T cell reactivity to different extracts is allergen specific and associated with different antigenic compounds. A large number of γδ TCR ligands and compounds/immunogens were previously identified as antigenic for human γδ T cells. This included broad and structurally diverse molecules such as major histocompatibility complex (MHC) class I-related molecules, nuclear proteins, heat shock proteins, lipids, and phosphoantigens, to name a few.[52,66] We cannot rule out that the γδ T cell activities measured to the allergen extracts could be related to a cellular stress response, although we find it unlikely since in the case of MO extract, γδ T cell reactivity was preserved even in the absence of non-CD3$^+$ populations.

Among specific antigens present in the MO extracts that may trigger γδ T cell activation, UL-16-binding protein-like transcript 1 (MULT-1), histocompatibility 60 (H60), and the retinoic acid early inducible-1 (Rae-1) α-ε family have been investigated for their ability to activate human γδ T cells.[67] In the case of CR extract, Bla g 1, one of the major allergens present, has been shown to contain many saturated lipids and phospholipids.[68] In TG extracts, exogenous pollen membrane lipids could be recognized by γδ T cells, as observed for pollen-derived phosphatidyl-ethanolamine.[18] Similarly, CD1a-restricted γδ T cell responses to lipid antigens from aerosolized HDM extracts have been described.[69] Further biochemical studies are required to map the antigens recognized by allergen-specific γδ T cells.

Since the AIM approach developed in this study allows for selective sorting of reactive cells, downstream applications such as RNA sequencing (RNA-seq) or single-cell RNA-seq (scRNA-seq) can be employed to further detail the features associated with allergen-specific γδ T cells, including γδ TCR profiling. Further assessment of the activation pathways and cytotoxic activities could also lead to improved diagnostics of the underlying pathobiology of allergic disease and elucidate the potential role of γδ T cells in AIT.

In summary, this study describes the development of a novel functional assay for the in-depth characterization of γδ T cells in allergy. In addition, our results indicate that allergen-specific γδ T cells display a highly Th1-polarized profile in allergic donors with potential immunotherapeutic application by rebalancing Th2-polarized responses in allergic settings.

Limitations of the study

The methodology developed herein allows the study of γδ T cell responses in response to a stimulus over short periods of culture. Therefore, it is unsuitable for directly quantifying or characterizing antigen-specific γδ T cells without stimulation. This methodology was also developed using isolated PBMCs, and compatibility using whole blood needs to be addressed. As with similar techniques developed for αβ T cells, the ICS and AIM techniques developed here for studying γδ T cells are also subject to the potential concern of bystander activation. However, we have demonstrated minimal bystander activation through several independent tests.

This study does not find any difference between sensitized and non-sensitized individuals, and the extracts used are highly complex, containing many types of molecules and potential antigens,[70,71] suggesting that γδ T cells can be reactive to several of their components. Therefore, a limitation of this study is the unknown nature of the antigen(s) being recognized in the allergen extracts and/or if, in fact, measured cells are responding to an antigen they have seen before. Further assessments of the individual components of allergen extracts by mass spectrometry and expansion of γδ T cells and assessment against those components in additional assays, such as ELISpot, would be helpful to support evidence that, in fact, AIM-assay-measured cells are activated by a previously specific response to that antigen.

In addition, transcriptomic analysis and assessment of TCR repertoire and clonality will elucidate if instead γδ T cell activation

involves cross-reactivity without previous antigen encounter or a superantigen-like activation.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Designing research studies, A. Sette, R.d.S.A., and E.D.Y.; investigation, R.d.S.A., E.D.Y., E.W., E.G., A. Sutherland, J.P., and V.S.; data analysis, E.D.Y. and R.d.S.A.; resources, N.K., K.K., and B.P.; manuscript writing, A. Sette, R.d.S.A., and E.D.Y.; supervision, A. Sette and R.d.S.A.; project administration, A.F.; funding acquisition, A. Sette and R.d.S.A.

DECLARATION OF INTERESTS

The authors have declared no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CD3 (AF700) (UCHT1)	Life Tech	Cat#: 56-0038-42; RRID: AB_10597906
anti-CD4 (APCef780) (RPA-T4)	Life Tech	Cat#: 47-0049-42; RRID: AB_1272044
anti-CD8 (PerCP-Cy5.5) (RPA-T8)	Life Tech	Cat#: 45-0088-42; RRID: AB_1582255
anti-CD14 (V500) (M5E2)	BD Biosciences	Cat#: 561391; RRID: AB_10611856
anti-CD19 (V500) (HIB19)	BD Biosciences	Cat#: 561121; RRID: AB_10562391
anti-TCR αβ (PE-DA594) (IP26)	Biolegend	Cat#: 306726; RRID: AB_2566599
anti-TCR δ1 (FITC) (TS8.2)	Life Tech	Cat#: TCR2730; RRID: AB_223624
anti-TCR δ2 (BV421) (B6)	BD Biosciences	Cat#: 743749; RRID: AB_2741717
anti-CD137 (APC) (4B4-1)	Biolegend	Cat#: 309810; RRID: AB_830672
anti-CD69 (BV605) (FN50)	BD Biosciences	Cat#: 562989; RRID: AB_2737935
anti-CD154 (PE) (24–31)	Biolegend	Cat#: 310806; RRID: AB_314829
anti-IFNγ (PerCP-Cy5.5) (4SB3)	Life Tech	Cat#: 45-7319-42; RRID: AB_10718246
anti-IL-4 (PE-Cy7) (MP4-25D2)	Biolegend	Cat#: 500824; RRID: AB_2126746
anti-IL-17 (FITC) (BL168)	Biolegend	Cat#: 512303; RRID: AB_961391
anti-IL-10 (PE-DA594) (JE53-19F1)	Biolegend	Cat#: 506812; RRID: AB_2632783
Live/Dead Viability (eF506/Aqua)	Invitrogen	Cat#: 65-0866-18; RRID: N/A
Biological samples		
Human blood samples	La Jolla Institute for Immunology	https://www.lji.org
Chemicals, peptides, and recombinant proteins		
Mouse extract	CliniSciences	https://www.clinisciences.com
Cockroach extract	La Jolla Institute for Immunology	https://www.lji.org
HDM Der p- and Der f-extracts	ALK-Abello A/S	https://www.alk.net
Timothy Grass extract	Greer Laboratories	https://www.stagrallergy.com
HDMAPP	Echelon Biosciences	https://www.echelon-inc.com
Mouse peptide pools	TC Peptide Lab	http://tcpeptidelab.com
Software and algorithms		
GraphPad Prism Version 9	GraphPad Software	https://www.graphpad.com
Microsoft Excel Version 16.16.27	Microsoft	https://www.microsoft.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact: Dr. Ricardo da Silva Antunes (rantunes@lji.org).

Materials availability

Materials used in this study will be made available to the scientific community upon request, and following execution of a material transfer agreement (MTA), by contacting R.d.S.A (rantunes@lji.org). Likewise, biomaterials archived from this study may be shared for further research with MTA.

Data and code availability

- The datasets generated and analyzed in this study will be shared by the lead contact upon reasonable request. Additional Supplemental Items are available from Mendeley Data at https://doi.org/10.17632/7tfy4jbxp9.1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The study cohort recruited for this study included 165 allergic patients. 153 donors were sensitized to a single allergen (33 MO, 48 CR, 48 HDM, and 24 TG) while 12 donors were sensitized for both MO and CR, defined by allergen-specific IgE titers of >0.35 kUA/l. 51 non-allergic healthy control subjects were used as control (Tables S1–S3). All donors were from San Diego, CA and provided informed consent with approval from the Institutional Review Board of La Jolla Institute for Immunology, La Jolla, CA (IRB Protocol no. VD-145). Each participant was assigned a study identification number with clinical information recorded. Clinical symptoms of allergy were collected by questionnaire-based survey and IgE-titers were determined from plasma using Phadia's ImmunoCAP assay (ThermoFisher Scientific, Waltham, MA). Adults of all races, ethnicities, ages, and genders were eligible to participate, the association of gender on the results of the study was not explicitly measured.

METHOD DETAILS

PBMC isolation

PBMCs were isolated from whole blood by density gradient centrifugation according to manufacturer instructions (Ficoll-Hypaque, Amersham Biosciences, Uppsala, Sweden) and cryopreserved for further analysis.

Extracts and peptide synthesis

Mouse extract synthesis was performed as previously described.[41] Briefly, mouse urine (mixed gender pooled, unfiltered) was purchased from CliniSciences (Nanterre, France), lyophilized and subsequently resuspended in PBS at 5.7 mg/mL (confirmed by BCA assay). CR extract from German cockroach fecal matter was manufactured in house at the La Jolla Institute for Immunology (La Jolla, CA, USA) using established protocols described elsewhere[72] and dissolved in PBS at 6.0 mg/mL. HDM Der p- and Der f-extracts were purchased from ALK-Abello A/S (Horsholm, Denmark), and TG extracts were purchased from Greer Laboratories (Lenoir, NC, USA), and were dissolved in PBS at 53.4 mg/mL and 5 mg/mL, respectively. For some experiments, extracts were subjected to fractioning with low molecular components (<3 kDa) separated from high molecular components (>3 kDa) by centrifugal filtration using Amicon Ultracel tubes (Merck Millipore, Darmstadt, Germany) with a cutoff of 3kDa. A total of 106 epitopes previously identified from mouse allergen extracts[41] were synthesized and used as a control in a small subset of experiments. Peptides were purchased from TC Peptide Lab (San Diego, CA) as crude material on a small (1 mg) scale. Individual peptides were resuspended, lyophilized, and then resuspended in DMSO to a final concentration of 2 mg/mL.

Activation-induced marker (AIM) and intracellular cytokine staining (ICS) assays

Evaluations of $\gamma\delta$ T cell responses were based on previously described Activation Induced Marker (AIM) *ex vivo* assays[37,40,49] utilizing CD137 (4-1BB), CD69, and CD154 (CD40L) markers, alone or combined with intracellular cytokine staining (ICS) for IFNγ, TNFα, IL-4, IL-5, IL−17, and IL-10 secretion. Briefly, for the CD137 and CD69 assay, PBMCs were thawed and rested overnight in a 96-well plate at 1×10^6 cells per well. 18–22 h later, cells were stimulated with extracts (10 μg/mL), HDMPP (10 μg/mL) or alternatively phorbol myristate acetate (PMA) and Ionomycin (Ion) (1 μg/mL) as positive controls, or medium alone as negative control. Alternatively, to evaluate cytokine responses, $\gamma\delta$ T cells were stimulated with MO and CR extracts in the presence of 1 μg/mL CD40 blocking antibody (Miltenyi Biotec, Auburn, CA, USA) and incubated at the final concentration of 1 μg/mL for 6 h in in 96-wells U bottom plates at 2×10^6 PBMCs per well. Golgi-Plug containing Brefeldin A (BD Biosciences, San Diego, CA) was added 3 h into the culture (1 μg/mL) as previously described.[73] After the incubation, cells were stained with surface markers for 30 min at 4°C followed by fixation with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) at 4°C for 10 min. Intracellular staining was performed at room temperature for 30 min after cells were permeabilized with saponin. All antibodies and their use in the different assays are shown in the key resources table. Data were acquired in a ZE5 4-laser cell analyzer (Bio-rad laboratories, Hercules, CA) and analyzed by FlowJo X Software (version 10) (Tree Star, Ashland, OR).

APC depletion and TCR blocking assessments

CD3-positive T cells were purified using the human T cell negative selection kit (EasySep™ Human T cell Isolation Kit, Stemcell Technologies, Vancouver, BC, Canada). APCs were separated from PBMCs in the negative flow-through using the human CD3 positive selection kit (EasySep™ Human CD3 Positive Selection Kit II, Stemcell Technologies, Vancouver, BC, Canada). Allergen-reactive T cell responses were detected from purified cells in the presence or absence of APCs using AIM assay. TCR blocking assessment experiments were performed by incubating human PBMCs with the TCR blocker dasatinib (Sigma-Aldrich, St. Louis, MO, USA) for 24 h in the presence of allergen extracts or LPS (Sigma). Allergen-specific $\alpha\beta$ or $\gamma\delta$ T cell responses were assessed using AIM assay.

Analysis of T cell responses

All responses measured by AIM or AIM in combination with ICS experiments were plotted and calculated by subtracting the specific stimuli by the background activation levels of controls (Culture media and DMSO for $\gamma\delta$ or $\alpha\beta$ T cells, respectively), except for Figures 1 and 2, and S4, where raw values of background and stimuli signals are plotted side-by-side for sense of comparison. Alternatively, responses were plotted as Stimulation Index (SI), calculated by dividing the counts of AIM + cells after specific stimulation

with the ones in the negative control. Criteria for positivity to a given extract were defined as follows: (1) The T cell reactivity needed to be significantly higher ($p < 0.05$) compared to background, as assessed by Student T-test, two-tailed, non-parametric; (2) A minimum response of $\geq$100 cells per 10^6 $\gamma\delta$ or $\alpha\beta$ T cells, and $\geq$10 cells per 10^6 of $CD3^+$ T cells after background subtraction; (3) The T cell reactivity observed needed to reach a SI $\geq$ 2, i.e. have a magnitude at least 2-fold higher than the background. The gating strategy utilized is shown in each respective figure and in Figure S6A.

QUANTIFICATION AND STATISTICAL ANALYSIS

Comparisons between allergic and non-allergic individuals were performed using the nonparametric two-tailed and unpaired Mann-Whitney tests. Wilcoxon or Kruskal-Wallis tests were performed for additional paired analysis as indicated in the respective figure. Prism 8.0.1 (GraphPad, San Diego, CA, USA) was used for these calculations. Values pertaining to significance and correlation coefficient (R) are noted in the respective figure, and $p < 0.05$ defined as statistically significant. The coefficient of variation (CV) was calculated as follows: CV=(Standard Deviation /Mean)×100. The Stimulation Index (SI) was calculated by dividing the count of AIM + cells after antigen stimulation with the ones in the negative control. All of the statistical details of experiments can be found in the figure legends and results, including the statistical tests used and sample sizes for each experiment.

Study approval

This study was approved by the Institutional Review Board of La Jolla Institute for Immunology (IRB protocol no. VD-145). Each participant provided informed consent and was assigned a study identification number with clinical information recorded.