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► To cite this version:

Augustin Belkadi, Céline Dietrich, François Machavoine, Jefferson Victor, Maria Leite-De-Moraes. $\gamma\delta$ T cells amplify *Blomia tropicalis* -induced allergic airway disease. *Allergy*, 2019, 74 (2), pp.395-398. 10.1111/all.13618 . hal-02324259

HAL Id: hal-02324259

<https://hal.science/hal-02324259>

Submitted on 5 Nov 2020

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$\gamma\delta$ T cells amplify *Blomia tropicalis*-induced allergic airway disease

Journal of Allergy and Clinical Immunology

Section designation: Letter to the Editor

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Disclosure of potential conflict of interest:

The authors declare that they have no relevant conflicts of interest.

Acknowledgements:

We are grateful to Momtchilo Russo and Michel Dy for discussions concerning *B. tropicalis*
and allergic mechanisms and Bernhard Ryffel for giving us the first TCR $\delta^{-/-}$ mice to start our
colony. We are indebted to the technical assistance of E. Panafieu and Rachel Rignaud and to

the help of Clélia Comte with the histology figures. Our thanks go also to the Cytology and Histology platforms of the INEM. This work was supported by grants from CNRS (Centre National de la Recherche Scientifique), INSERM (Institut National de la Santé et de la Recherche Médicale), Université Paris Descartes and Legs Poix (Chancellerie des universités de Paris).

Authorship Contributions:

M.L.M conceived and designed the experiments.
A.B., C.D., F.M., and J.R.V. performed the research.
A.B., C.D., J.R.V., and M.L.M. analysed the data.
M.L.M. wrote the manuscript

Text Count

Word count in the body of manuscript: 997
Figures: 2 (+ 5 supplementary)
Tables: 1 supplementary

Key messages (2-3 independent bulleted statements)

- IL-4-producing V γ 1⁺ $\gamma\delta$ T cells promote allergic airway inflammation in a *Blomia tropicalis* model of asthma
- Human peripheral blood $\gamma\delta$ T cells are activated in atopic *B. tropicalis* subjects compared to non-atopic donors.

Key Words

Blomia tropicalis; $\gamma\delta$ T cells, atopy; asthma; airway hyperreactivity; lung, IL-4, patients

Short title: $\gamma\delta$ T cells and *Blomia tropicalis* allergic immune responses

ABBREVIATIONS

AHR: airway hyperreactivity
BALF: bronchoalveolar lavage fluid
WT: wild type

To the Editor:

Allergic asthma is a chronic and heterogeneous immunological disease characterized by airway inflammation, hyper-IgE production, mucus hypersecretion and airway hyperreactivity (AHR). The most studied house dust mites (HDM) respiratory allergens belong to *Dermatophagoides pteronyssus* and *Dermatophagoides farinae* mite species. However, the storage mite *Blomia tropicalis* represents an important allergenic source and sensitization to its allergens are commonly associated with typical clinical manifestations of allergy, including asthma (1-3). Although these mites may coexist in tropical and subtropical regions, *B. tropicalis* is the major allergen in some countries (4, 5). A limited number of reports have focused on the allergic processes involved in the immune responses triggered by *B. tropicalis* (3, 6, 7). Here, we used allergic asthma murine models and *ex vivo* analysis of *B. tropicalis* allergic subjects to better explore the role of immune cells in allergic responses obtained in the context of *B. tropicalis* exposure.

Mice received *B. tropicalis* allergens exclusively administered intranasally (without adjuvant) for both sensitization and challenge phases (Appendix S1). This model reproduced the major allergic asthma hallmarks, namely enhanced BALF cell counts accompanied by airway eosinophilia and airway hyperresponsiveness (AHR) (Figure 1A and B). We addressed the involvement of IL-4, as a major Th2 cytokine by comparing lung inflammation in IL-4-deficient (IL-4^{-/-}) and wild-type (WT) mice. The lack of IL-4 resulted in the absence of the typical airway eosinophilia and AHR observed in WT mice following *B. tropicalis* sensitization and challenge (Figure S1A and B). These results show that IL-4 is required for *B. tropicalis* allergen-induced asthma in our model.

Distinct immune cells, such as iNKT cells and $\gamma\delta$ T cells, may be capable to secrete IL-4 and favour asthmatic inflammatory Th2-cytokine-dependent responses (8, 9). As previously described, iNKT cells can be divided into three major subsets, iNKT1 (IFN- γ and

IL-4 producers), iNKT2 (IL-4 producers) and iNKT17 (IL-17 producers) (10). These subsets were present in the lung of *B. tropicalis* treated mice (Figure S2A). No major differences were observed concerning their ability to secrete IL-4, IL-13 or IFN γ but the frequency of IL-17-producing iNKT cells was enhanced when compared to controls (Figure S2A). However, this enhanced frequency was not critical for airway inflammation since iNKT cell-deficient mice presented similar airway eosinophilia than WT mice (Figure S2B).

Concerning $\gamma\delta$ T cells, including the V γ 1.1⁺ (hereafter mentioned as V γ 1⁺) $\gamma\delta$ T cell subset, we found that their number were higher in the lung of *B. tropicalis*-treated than in NaCl-treated mice (Figure S3). It is noteworthy that airway eosinophilia and AHR were significantly reduced in *B. tropicalis*-sensitized and -challenged TCR $\delta^{-/-}$ mice when compared to WT controls (Figure 1C, 1D). Further, IgE levels in the serum, Mucin-5 mRNA expression and mucus deposition in the lung were also impaired in TCR $\delta^{-/-}$ compared to WT mice (Figure S4). Previous reports have shown that the influence of $\gamma\delta$ T cells on allergic responses may be complex (9). Here, we focused our analysis on V γ 1⁺ and V γ 1⁻ $\gamma\delta$ T cell subsets (Figure S5A). Among $\gamma\delta$ T cells, IL-4 and IL-13 were mainly produced by the V γ 1⁺ $\gamma\delta$ T cell subset in the lungs of the asthmatic mice (Figure S5B and C). Of note, the frequency of IL-4⁺ and IL-13⁺ among gated V γ 1⁺ $\gamma\delta$ T cells from *B. tropicalis*-treated mice was higher than in controls (Figure S5B). These cells also secreted higher levels of IFN γ and IL-17 in asthmatic mice (Figure S5B). In contrast, the percentage of IL-4⁺, IL-13⁺, IL-17⁺ and IFN γ ⁺ among gated V γ 1⁻ subset was similar in mice treated with *B. tropicalis* and controls (Figure S5B and C). These findings indicating that cytokine-producing V γ 1⁺ $\gamma\delta$ T cells may be critical for this inflammatory allergic response.

The enhanced frequency of IL-4-producing V γ 1⁺ $\gamma\delta$ T cells in the lung of *B. tropicalis*-treated mice (Figure S5B) led us to the question whether airway eosinophilia and AHR could

be restored by transferring fully competent $V\gamma 1^+ \gamma\delta$ T cells to *B. tropicalis*-sensitized and -challenged $TCR\delta^{-/-}$ mice. This was the case since the adoptive transfer of 25 000 $V\gamma 1^+ \gamma\delta$ T cells from WT mice one hour before the first challenge restored airway inflammation, particularly for airway eosinophilia, and AHR in $TCR\delta^{-/-}$ mice (Figure 1E and F). In contrast, adoptive transfer of $V\gamma 1^+ \gamma\delta$ T cells from $IL-4^{-/-}$ mice had no effect (Figure 1E and F). The failure to develop an effective AHR response in $TCR\delta^{-/-}$ mice and the inability of $V\gamma 1^+ \gamma\delta$ T cells from $IL-4^{-/-}$ mice to restore AHR can be ascribed to the lack of IL-4 production by $V\gamma 1^+ \gamma\delta$ T cells, which compromises their contribution to asthmatic symptoms. Thus, IL-4-producing $V\gamma 1^+ \gamma\delta$ T cells are required for airway inflammation and AHR in our *B. tropicalis* asthma model.

We further addressed the relevance of $\gamma\delta$ T cells to atopic immune process observed in *B. tropicalis* allergic subjects. We obtained blood samples from subjects clinically classified as atopic or non-atopic (no allergic responses to any tested allergen) to *B. tropicalis* allergens (Table S1). The frequency of peripheral blood $\gamma\delta$ T cells was significantly lower in *B. tropicalis* atopic than non-atopic individuals (Figure 2A and S6). However, these cells were more activated in atopic subjects, as they spontaneously produced higher levels of $IFN\gamma$ and IL-4 than those of non-atopic donors (Figure 2B, 2C and S6). The frequency of IL-17-producing $\gamma\delta$ T cells was similar in atopic and non-atopic subjects (Figure 2D and S6). Overall, these observations suggest that $\gamma\delta$ T cells may be involved in immune allergic processes in *B. tropicalis* atopic patients. Further studies are required to determine the major blood $\gamma\delta$ T cell subsets activated in these patients.

This study has limitations since the precise nature of the stimuli (*B. tropicalis* antigens or cytokines) required to induce IL-4 production by $\gamma\delta$ T cells and the analysis of these lymphocytes in BALF from subjects susceptible to *B. tropicalis* remain to be determined.

However, our findings provided many insights into how $\gamma\delta$ T cells may contribute to immune allergic responses triggered by *B. tropicalis*. First, $\gamma\delta$ -deficient mice provided unequivocal evidence that the absence of $\gamma\delta$ T cells profoundly impairs the allergic asthma symptoms induced by *B. tropicalis* sensitization and challenge under defined experimental conditions. Moreover, airway eosinophilia and AHR in response to *B. tropicalis* were achieved only when V γ 1⁺ $\gamma\delta$ T cells from WT, but not from IL-4^{-/-}, mice were adoptively transferred to $\gamma\delta$ -deficient mice. Consistent with this result, $\gamma\delta$ T cells were activated in *B. tropicalis* atopic patients, as they produced higher levels of IFN γ and IL-4 than non-atopic donors. In conclusion, our results suggest an unanticipated role for $\gamma\delta$ T cells in allergic immune responses induced by *B. tropicalis*.

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LEGENDS

Figure 1: $\gamma\delta$ T cells were required for asthma symptoms induced by *B. tropicalis*. (A, C, E) The number of total cells (Total), monocyte (Mono), eosinophil (Eosino), neutrophil (Neutro) and lymphocyte (Lympho) were determined in BALF of wild-type (WT) or $\text{TCR}\delta^{-/-}$ asthmatic (*B. tropicalis*) or non-asthmatic (NaCl) mice (n=7 to 20). (B, D, F) Lung resistance (Rtr) was measured 24h after the last *B. tropicalis* challenge or controls (NaCl) (n=7 to 15). (E, F) BALF cell counts (E) and lung resistance (Rtr) were measured 24h after the last *B. tropicalis* challenge of WT, $\text{TCR}\delta^{-/-}$, $\text{TCR}\delta^{-/-}$ mice adoptively transferred with sorted $\text{V}\gamma 1^{+}$ $\gamma\delta$ T cells from WT or $\text{IL-4}^{-/-}$ mice (n=5 to 10). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Figure 2. $\gamma\delta$ T cells were activated in *B. tropicalis* atopic patients. (A) Percentages of $\gamma\delta$ T cells in PBMC from non-atopic versus *B. tropicalis* atopic subjects. (B, C, D) Frequency of spontaneous $\text{IFN}\gamma^{+}$ (B), IL-4^{+} (C) and IL-17A^{+} (D) cells among gated $\gamma\delta$ T cells from non-atopic versus *B. tropicalis* atopic subjects.

Table S1: Characteristics of the adults included in the study

	Non-atopic	Bt-Atopic	<i>p</i>
Number	15	13	
Age, years (mean $\pm$ SE)	30.8 $\pm$ 2.1	29.6 $\pm$ 2.8	0.41
Sex (male/female)	7/8	7/6	0.83
SPT results (n/%)			
<i>Blomia tropicalis</i>	0/0	13/100	
<i>Dermatophagoides pteronyssinus</i>	0/0	9/69	
<i>Dermatophagoides farinae</i>	0/0	8/61	
<i>Aspergillus fumigatus</i>	0/0	5/38	
<i>Canis familiaris</i>	0/0	5/38	
<i>Alternaria alternata</i>	0/0	4/30	
<i>Penicillium notatum</i>	0/0	4/30	
<i>Felis domesticus</i>	0/0	2/15	
<i>Cladosporium herbarum</i>	0/0	1/8	
<i>Periplaneta americana</i>	0/0	0/0	
Others			
Clinical manifestation (past 6 mo)	0/0	13/100	
Regular use of antihistamines	0/0	13/100	
SPT - Skin prick test; mo - months			

$\gamma\delta$ T cells amplify *Blomia tropicalis*-induced allergic airway disease

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METHODS

Mice and reagents

Eight to ten-week-old specific pathogen-free C57BL/6J, IL-4^{-/-} and TCR δ ^{-/-} mice were bred in our facility. All animal experiments were carried out according to the guidelines for care and use of animals approved by the French Institutional Committee (APAFIS#4105-201511171831592).

Airway allergen sensitization and challenge model.

To induce allergic airway inflammation, we adapted a previously described protocol (1). In brief, mice were sensitized and challenged by intranasal administrations of *B. tropicalis* extracts (Greer laboratories, USA) at day 0 (D0) with 100 μ g/mouse then at D7, D9, D11, D14 and D16 with 50 μ g/mouse.

Twenty-four hours after the last challenge, mice were anesthetized with a mixture of ketamine (150 mg/kg) and xylazine (400 μ g/kg) and their tracheas were cannulated (tracheostomy with ligation). FlexiVent apparatus (SCIREQ) was used to access airway-specific resistance (Rn, tidal volume of 10 ml/kg at a respiratory rate of 150 breath/min in response to increasing doses of aerosolized acetyl- β -methylcholine chloride (methacholine; Sigma-Aldrich). Assessments were performed at least three times and the maximum R value obtained after each dose of methacholine was used for the measure.

Airway inflammation was assessed in cytospin preparations of cells in bronchoalveolar lavage fluid (BALF, 3 x 0.5 mL washes with PBS) that were stained with May-Grünwald/Giemsa (Merck). For some experiments, BALF cells were also analysed by flow cytometry.

Adoptive transfer

In some experiments, 25 x 10³ electronically sorted V γ 1⁺ or V γ 1⁻ $\gamma\delta$ T cells were adoptively transferred intravenously (i.v.) to *B. tropicalis* immunized mice 1h before the first challenge (D7). Mice were then further challenged at at D7, D9, D11, D14 and D15 with 50 μ g/mouse, as described above.

Leucocytes from lung tissues

Lung tissues were cut into pieces using a GentleMACS Dissociator (Miltenyi Biotec) and treated with collagenase type 4 (Thermo Fischer Scientific) plus DNase I (Roche). The lymphocyte-enriched fraction was collected at the 35-70% interface of Percoll gradients (GE Healthcare). Cells were immediately stained or stimulated for 4h with 10⁻⁸ M PMA and 1 μ g ml⁻¹ ionomycin, in the presence of 10 μ g ml⁻¹ brefeldin A (all from Sigma-Aldrich).

Immunofluorescence.

Murine cells were incubated with CD1d-PBS57-APC tetramers, anti-TCR β -APCeFluor780, anti-V γ 1.1-PE and anti-TCR $\gamma\delta$ -BV605 (Biolegend). Fixable viability dye was used to exclude dead cells (ThermoFischer Scientific). For intracellular staining, cells were further fixed with 4% PFA, washed, and permeabilized with 0.5% saponin (Sigma-Aldrich), and then incubated

with anti-IL-17-PerCPCy5.5, anti-IL-4-PE-Cy7, anti-IFN- γ -V450, anti-anti-IL-13-PE-eF610 or isotype control (eBiosciences). The cells were washed and fluorescence was detected using a LSRFortessa (Becton Dickinson) and further analysed using the FlowJo 10.4.1 software (Tree Star).

$\gamma\delta$ T cell sorting.

Splenocytes from WT or IL-4^{-/-} mice, isolated as previously described (2, 3), were labelled with anti-V γ 1-PE and anti-TCR $\gamma\delta$ -BV605, as described above, and then sorted using a FACSARIA cell sorter (Becton Dickinson).

mRNA expression.

RNAs were extracted using the RNeasy Plus Minikit (Qiagen) including a DNase treatment. Then RNA was reverse transcribed using the High Capacity RNA-to-cDNA Kit (ThermoFisher Scientific), according to the manufacturer's instructions. Primers and probes for real-time PCR were provided by ThermoFisher Scientific under references: Mucin 5b (Muc5b): Mm00466391_m1 and HPRT: Mm01545399_m1. All reactions were performed in triplicate with TaqMan® Fast Advanced Master Mix according to the supplier's instructions for a Step One Plus apparatus (ThermoFisher Scientific). All data were normalized to the internal standard, namely HPRT expression in each sample, and expressed as relative expression using the $\Delta\Delta C_t$ method versus the reference sample.

Histology of the airways.

Lungs were fixed with 10% formalin via the trachea, removed and stored in 10% formalin. Lung tissues were embedded into paraffin and 3 μ m sections were stained with periodic acid Schiff (PAS).

Patient samples

Blood samples were collected from subjects who were previously clinically classified as atopic or non-atopic individuals and voluntarily subjected to a skin prick test (SPT) to confirm the atopic state. These individuals were classified into two groups: as Bt- atopic individuals (clinically allergic and reactive to *Blomia tropicalis* extract, with co-reaction to at least one allergen), and non-atopic individuals (without any clinical allergy symptoms and not reactive to any tested allergen). Additional information about these individuals is shown in table 1.

Each sample of peripheral blood mononuclear cells (PBMCs) was provided from a different donor. The ethics committees at the HCor and the School of Medicine at the University of São Paulo approved this study (CAAE: 15507613.4.0000.0060).

Skin prick test (SPT) and blood sample collection

The SPT were performed in accordance with European standards (4) with an adapted panel of allergens that included the profile of Brazilian allergens (i.e., *Blomia tropicalis*, *Canis familiaris*, *Periplaneta americana*, *Aspergillus fumigatus*, *Penicillium notatum*, *Alternaria alternata*, *Cladosporium herbarum*, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Felis domesticus* - IPI ASAC), as previously described (5).

Briefly, one drop of each allergen extract or controls was applied to the volar forearm and a superficial skin puncture was made using a hypodermic needle (Alko, Brazil). After 15 minutes, the results were considered positive when wheals measure reach a diameter of 3 mm greater than that of the negative control. We excluded patients who used antihistamines, glucocorticosteroids or certain other systemic drugs that can influence the SPT results within 15 days before the test or with severe eczema or dermatographism.

Human flow cytometry

Flow cytometry using human PBMCs to evaluate phenotype and spontaneous intracellular cytokine production were performed as previously described (6, 7). Briefly, PBMC separations were performed using Ficoll-Paque Plus (GE Healthcare) after centrifugation and suspensions of PBMCs were phenotypic evaluated *ex vivo* or cultured to intracellular cytokine production evaluation. PBMCs were stained with mouse anti-human $\gamma\delta$ TCR-FITC, CD161-PECy5 or isotype control antibodies (BD Pharmingen).

To evaluate intracellular cytokine production, PBMCs were cultured in RPMI 1640 medium containing 10% FBS and 1 μ g/mL of Brefeldin A (Sigma-Aldrich) for 24 hours without additional stimulus. Cultured PBMCs were then washed, fixed with formaldehyde and stained with mouse anti-human $\gamma\delta$ TCR-FITC, IL-17A-Alexa700, IL-4-PECy7, IFN- γ -HorizonV450 or isotype control antibodies (BD Pharmingen) in 100 μ L of PBS containing 0.05% saponin. Thirty minutes later, PBMCs were washed, fixed, acquired using a LSRI Fortessa cytometer (BD Biosciences, USA), and analysis was performed using FlowJo software 10.1 (Tree Star).

Statistics

Data are expressed as means $\pm$ SEM. The AHR values were analysed with repeated-measures 2-way ANOVA followed by Bonferroni correction as a post-hoc test. All other values were analysed with Mann-Whitney U test. Results were considered significant at a *P* value of 0.05 or less (**p*<0.05; ***p*<0.01; ****p*<0.001). Data were analyzed using GraphPad Prism version 6 (GraphPad Software).

LEGENDS

Figure S1: IL-4-producing cells were required for asthma symptoms induced by *B. tropicalis*. (A) The number of total cells (Total), monocyte (Mono), eosinophil (Eosino), neutrophil (Neutro) and lymphocyte (Lympho) were determined in BALF of wild-type (WT) or IL-4^{-/-} asthmatic (*B. tropicalis*) mice. (B) Lung resistance (Rtr) was measured 24h after the last *B. tropicalis* challenge or controls (NaCl) (n=7 to 15).

Figure S2: iNKT cells were not required for asthma symptoms induced by *B. tropicalis*. (A) A representative FACS profile showing the gating strategy used to identify iNKT (CD1d tetramer⁺TCR β ⁺) cells and the percentage of IFN γ , IL-4, IL-13 and IL-17A positive cells, and their respective isotype controls, among gated iNKT cells in the lungs of WT asthmatic (*B. tropicalis*) or non-asthmatic (NaCl) mice. (B) The number of total cells (Total), monocyte (Mono), eosinophil (Eosino), neutrophil (Neutro) and lymphocyte (Lympho) were determined in BALF of wild-type (WT) or iNKT (J α 18^{-/-})-deficient asthmatic (*B. tropicalis*) mice. (n=5 to 7).

Figure S3: $\gamma\delta$ T cells and the V γ 1⁺ $\gamma\delta$ T cell subset were enhanced in the lung of *B. tropicalis* asthmatic mice. (A) A representative FACS profile showing the gating strategy used to identify TCR $\gamma\delta$ ⁺ and V γ 1⁺ $\gamma\delta$ T cells. (B) The number of total $\gamma\delta$ T cells and of the V γ 1⁺ $\gamma\delta$ T cell subset were determined in the lungs of wild-type asthmatic (*B. tropicalis*) or non-asthmatic (NaCl) mice (n=5 to 10).

Figure S4: Impaired seric IgE and mucus production in the lung of TCR δ ^{-/-} *B. tropicalis* asthmatic mice. (A) Total IgE levels in the serum of WT and TCR δ ^{-/-} mice following *B. tropicalis* or NaCl administration (B) Mucin-5 mRNA expression assessed by quantitative RT-PCR in the lung of WT and TCR δ ^{-/-} mice following *B. tropicalis* or NaCl administration.

Data are representative of two experiments. (C) Representative PAS-stained lung histology sections of controls (NaCl) and *B. tropicalis*-sensitized and -challenged WT and TCR $\delta^{-/-}$ mice. Following *B. tropicalis* administration, the airways of WT mice contained more PAS-stained mucus-producing cells than TCR $\delta^{-/-}$ mice.

Figure S5. Distinct cytokine production by lung V $\gamma 1^{+}$ and V $\gamma 1^{-}$ $\gamma\delta$ T cell subsets. (A) Flow cytometric gating strategy used to identify TCR δ^{+} V $\gamma 1^{+}$ and TCR δ^{+} V $\gamma 1^{-}$ $\gamma\delta$ T cell subsets and the frequency of these cells in the lung of *B. tropicalis*- versus NaCl-treated mice. (B, C) A representative FACS profile showing the percentage of IFN γ , IL-4, IL-13 and IL-17A positive cells among gated TCR δ^{+} V $\gamma 1^{+}$ (B) and TCR δ^{+} V $\gamma 1^{-}$ (C) $\gamma\delta$ T cell subsets and their frequency in the lungs of WT asthmatic (*B. tropicalis*) or non-asthmatic (NaCl) mice. * $p < 0.05$. (D) FACS profile represents the respective IL-13 versus IL-4 and IL-17A versus IFN- γ isotype controls.

Figure S6. $\gamma\delta$ T cells were activated in *B. tropicalis* atopic patients. (A) Gate strategy to obtain viable lymphocytes. (B) A representative FACS profile showing the gating strategy used to identify TCR $\gamma\delta^{+}$ cells and the percentage of IFN γ , IL-4, IL-13 and IL-17A positive cells among gated TCR $\gamma\delta^{+}$ cells.

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Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6