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High-dose bee venom exposure induces similar tolerogenic B cell responses in patients and healthy beekeepers

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Abstract

Background: The involvement of B cells in allergen tolerance induction remains largely unexplored. This study investigates the role of B cells in allergen tolerance induction to high-dose allergen exposure, and to compare B cell responses between allergic patients receiving allergen immunotherapy (AIT) and naturally exposed healthy beekeepers before and during the beekeeping season.

Methods: Circulating B cells were characterized by flow cytometry. PLA-specific B cells were identified using dual color staining with fluorescently labeled phospholipase A2 (PLA). Expression of regulatory B cell-associated surface markers, interleukin-10, chemokine receptors and immunoglobulin heavy chain isotypes was measured. Specific and total IgG1, IgG4, IgA and IgE from plasma as well as culture supernatants of PLA-specific cells were measured by ELISA.

Results: Strikingly similar responses were observed in allergic patients and beekeepers after venom exposure. Both groups showed increased frequencies of plasmablasts, PLA-specific memory B cells and IL-10-secreting $CD73^{-}CD25^{+}CD71^{+}$ B_R1-cells. PLA-specific IgG4-switched memory B cells expanded after bee venom exposure. Interestingly, PLA-specific B cells showed increased CCR5 expression after high-dose allergen exposure while CXCR4, CXCR5, CCR6 and CCR7 expression remained unaffected.

Conclusions: This study provides the first detailed characterization of allergen-specific B cells before and after bee venom tolerance induction. The observed B cell responses in both VIT-treated patients and naturally exposed beekeepers suggest a similar functional immunoregulatory role for B cells in allergen tolerance in both groups. These findings can be

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investigated in other AIT models to determine their potential as biomarkers of early and successful AIT responses.

Keys words: bee venom allergy, allergen specific immunotherapy, immune tolerance, IgG4, CCR5.

Short title: B cell responses in allergen tolerance induction.

Abbreviations:

AIT: Allergen-specific immunotherapy

BCR: B cell receptor

B_R1: B regulatory 1

Breg: Regulatory B

PLA: phospholipase A2

PLA^+: PLA-specific

PLA^-: PLA-non-specific

Th: T helper

T_R1: T regulatory 1

Treg: Regulatory T

VIT: venom immunotherapy

INTRODUCTION

Allergen-specific immunotherapy (AIT) is currently the only available treatment that can lead to allergen tolerance. AIT can result in a reduction of allergic symptoms, disease severity and decreased medication use (1). Insect venom immunotherapy (VIT) has been proven effective for preventing further allergic reactions to insect stings (2). Allergic patients

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receiving VIT as well as healthy non-allergic beekeepers, represent good human *in vivo* models to study the mechanisms of immune tolerance induction to allergens. Within a week after the initial bee venom exposure the frequency of allergen-specific IL-10-producing Tr1 increases (3, 4). Venom-specific Th1 and Th2 cells switch toward IL-10-secreting T_R1 cells, which suppress T cell responses through various mechanisms (3-5). IL-10 has a wide range of immunosuppressive functions including suppression of proinflammatory cytokine production, suppression of effector T cell responses as well as augmentation of IgG4 production and suppression of IgE production by B cells (6-8).

AIT also promotes IL-10 secreting B regulatory 1 (B_R1) cells, which can regulate immune responses through suppression of antigen-specific $CD4^+$ T cell proliferation and production of non-inflammatory IgG4 antibodies. Allergen-specific IgG4 can interfere with allergen-mediated IgE crosslinking, thereby preventing mast cell and basophil degranulation (9, 10). Furthermore, AIT can stimulate somatic hypermutation of allergen-specific B cells, leading to generation of high-affinity antibodies (11). In response to AIT, typically a transient increase in circulating specific IgE is observed, followed by gradual decrease over subsequent months (12). In contrast, circulating specific IgG4 increases during AIT (13). However, the correlation between serum IgG4 and clinical outcome of AIT remains controversial, and allergen-specific B cell responses have not been thoroughly studied (1). Interestingly, a recent study demonstrated a positive correlation between serum histamine levels and venom-specific IgG4 antibodies in beekeepers, associating high histamine levels in serum with a tolerant phenotype (14).

Allergen-specific B cells can be detected in circulation at very low frequencies (11). Accurate detection of allergen-specific B cells therefore requires a thorough exclusion of non-specific staining. It has been demonstrated that small fractions (<0.05%) of B cells express a B cell receptor (BCR) that specifically recognizes fluorescent dyes such as PE or APC (15). Therefore detection of allergen-specific B cells without inclusion of dye-specific B cells will be more accurate when cells are stained with antigens labeled with two structurally unrelated fluorescent dyes.

Chemokine receptors play a key role in the regulation of immune responses by coordinating chemotaxis of immune cells. B cells express a range of chemokine receptors including CXCR4, CXCR5, CCR6 and CCR7 (16). CCR5 is not widely expressed on B cells but was of interest because plays an important role in the functionality of Treg cells in immune tolerance (17-19). CXCR5 and CCR7 primarily regulate cell trafficking in lymphoid tissues, CXCR4 facilitates bone marrow homing and trafficking of B cells in lymph nodes, and CCR5 and CCR6 promote homing to peripheral sites of inflammation (16).

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There are still many open questions regarding the role of allergen-specific B cells in the induction of immune tolerance to allergens. In this study, we investigated the effect of VIT as well as natural tolerance induction on allergen-specific B cell responses. We used dual-color fluorescent staining to identify PLA-specific B cells. Using this method, we could isolate PLA-specific B cells and confirm their antigen specificity. Moreover, we used flow cytometry to characterize PLA-specific B cells. This approach revealed that allergen-specific memory B cells expand in response to high-dose allergen exposure both in patients as well as healthy individuals. Furthermore B cells developed an immunoregulatory phenotype, characterized by elevated production of the immunoregulatory mediators IL-10 and IgG4, and increased CCR5 expression, which may facilitate their migration to site of inflammation, where they can exert their regulatory function.

METHODS

Subjects

Peripheral blood was obtained from 14 bee venom allergic patients, who underwent VIT and 15 beekeepers (before and during the beekeeping season). Three bee venom allergic patients received rush VIT and 11patients received ultra rush VIT. Details on the immunotherapy protocol are described elsewhere (20, 21). Blood samples were collected at baseline before subcutaneous VIT, and at week 7 in rush VIT, and 10 weeks after the start of ultra rush VIT. Samples were collected from beekeepers at baseline before the start of the beekeeping season (February to March), and after multiple bee stings during beekeeping season (May to October). Tables S1 and S2 list the detailed characteristics of patients and beekeepers. All participants gave written informed consent. This study was approved by the Ethical Commission of the Canton of Graubünden, Switzerland, the Ethical Commission of the Canton of Bern, Switzerland, and the Ethical Commission of the Medical University of Bialystok, Poland.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were stained with Fixable Viability Dye eFlour780 (eBiosciences, San Diego, CA) according to manufacturer instructions. After that, cells were stained with antibodies against surface markers. The antibodies used for surface staining are listed in Table S3. Cells were analyzed and/or

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sorted with a FACSAria III (Beckton Dickinson, Franklin Lakes, NJ).

For the detection of IL-10-producing B cells, $2x10^6$ PBMC were stimulated for 48 hours with 1 µM of the TLR9 ligand CPG2006 (Microsynth, Balgach, Switzerland) in complete RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO). Then PBMC were stimulated for two hours with 20 ng/ml PMA and 1 µg/ml ionomycin followed by two hours stimulation with 10 µg/ml brefeldin A (all from Sigma-Aldrich, St. Louis, MO). Surface staining was performed, cells were washed, fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences) and stained with anti-IL-10-PerCP/Cy5.5 (clone JES3-9D7, Biolegend, San Diego, CA). Samples were analyzed and sorted with a FACSAria III (Beckton Dickinson, Franklin Lakes, NJ). Data were analyzed using Kaluza software (Beckman Coulter).

Antigen-specific B cells

The major bee venom allergen PLA was used as a representative allergen to identify bee venom-specific B cells. Purified PLA from honeybee venom (Sigma-Aldrich, St. Louis, MO) was labeled with Lightning-Link AF647 Rapid Conjugate System or Lightning-Link PE/Cy7 Tandem Conjugate Kit (Both from Innova Biosciences, Cambridge, UK). The conjugated PLA constructs were titrated for optimal concentration for staining. PLA-specific B cells were defined as $CD19^+CD27^+IgM^-$ (class-switched memory B cells) and double positive for PLA-AF647 and PLA-PE/Cy7. As a negative control, cells were incubated with an excess of non-labeled PLA for 15 minutes in the dark at 4°C before adding the conjugated PLA (Fig. S1).

PLA-specific antibody detection

To demonstrate the specificity of the allergen-specific B cell staining, PLA^+ and PLA^- memory B cells were sorted and cultured in 96 well plates in Iscove's Modified Dulbecco's Medium (Sigma-Aldrich) with 10^5/ml irradiated CD40L L cells (22) and rIL-21 (25ng/ml) for 72 hours at 37 °C. Supernatants were collected and we used ELISA to measure secreted PLA-specific immunoglobulins. 5µg/ml PLA was coated to a Nunc Maxisorb microtiter plate (Thermo Scientific, Waltham, MA) and then blocked with blocking buffer (PBS pH 7.4, 1% BSA, 0.05% Tween 20). Cell culture supernatant was incubated for 2 hours at room temperature. The following detection antibodies were used: mouse anti-human IgG4 (clone RJ4, Bionostics, Devens, MA), mouse anti-human IgG1 (BD Pharmingen, San Diego, CA), mouse anti-human IgG-PO (Jackson Laboratory, Bar Harbor, MA) followed by incubation

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with peroxidase-conjugated goat anti-mouse IgG (Thermo Scientific, Waltham, MA). Tetramethylbenzidine (TMB) substrate was used to develop the assay. The reaction was stopped by 2M sulfuric acid and OD450 nm values were measured by a Mithras LB 940 spectrophotometer (Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad Software, California, USA), and Wilcoxon matched pairs test was used for assessment of statistical significance. Statistically significant results are indicated on the graphs and figure legends.

RESULTS

Frequency of PLA-specific class-switched memory B cells increases in response to high-dose allergen exposure

To determine whether VIT and natural exposure to bee venom induced activation and expansion of allergen-specific B cells, we measured the frequency of PLA-specific class-switched memory B cells in PBMC before and during VIT in allergic patients and before and during the beekeeping season in beekeepers. PLA-specific class-switched memory B cells were identified as $CD19^{+}CD27^{+}IgM^{-}PLA^{+}$ (gating strategy of PLA-specific B cells is shown in Fig.S1). The frequency of circulating PLA-specific memory B cells was significantly increased during VIT compared to baseline (0.13 % ± 0.07 to 0.23 % ± 0.09) (Fig 1A). Similarly, the frequency of PLA-specific class-switched memory B cells was significantly increased (0.18 % ± 0.01 to 0.30 % ± 0.02) in the beekeeper blood samples that were collected after multiple stings during bee keeping season compared to the samples taken before the start of the season (Fig 1B). The percentage of PLA-specific class-switched memory B cells ($CD19^{+}CD27^{+}IgM^{-}PLA^{+}$) was higher in naturally exposed beekeepers than in the VIT group, but this difference was not statistically significant. The frequencies of PLA-specific, non-class-switched memory B cells ($CD19^{+}CD27^{+}IgM^{+}PLA^{+}$) showed a small but significant reduction during VIT (0.33% ±0.04 to 0.24±0.04) but were not significantly changed in beekeeper samples (Fig. S2).

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To confirm the specificity of the method for identification of PLA-specific B cells, we purified PLA-specific class-switched memory B cells (representing 0.01% - 0.60% of class-switched memory B cells) (Fig 1). PLA-specific and control ($CD19^+CD27^+IgM^-PLA^-$) cells were sorted by flow cytometry and cultured with CD40L and rIL-21. Secreted immunoglobulins were measured by ELISA. There was no significant difference in total IgG production between PLA-specific and control cells (Fig. S3). The amount of secreted PLA-specific IgG1 and IgG4 was significantly higher for PLA-specific cells compared to control cells (Fig. S3). These data confirm that our method of using dual-color surface staining with fluorescently labeled PLA can be used to detect and purify PLA-specific B cells.

Plasmablast frequency increases after high-dose allergen exposure

Plasmablasts secrete immunoglobulins, but continue to proliferate and can be found in circulation. Human plasmablasts can be identified using surface markers CD19, CD27 and CD38 (23). In bee venom allergic patients, the frequency of circulating plasmablasts ($CD19^+CD27^{hi}CD38^{hi}$) increased significantly during VIT (0.57% ± 0.09 to 0.82% ± 0.13) (Fig 2A). In the beekeeper group, there was also a significant increase in plasmablast frequency (0.29% ± 0.04 to 0.44% ± 0.06) after multiple bee stings during the season (Fig 2B).

IL-10-secreting Breg cells expand after high-dose allergen exposure

In order to detect IL-10-producing regulatory B cells, we stimulated PBMC with the TLR9 ligand CPG2006 for 48 hours before flow cytometric analysis. It was previously demonstrated that IL-10-producing B cells are enriched among $CD73^-CD25^+CD71^+$ B cells (24). We therefore investigated $CD73^-CD71^+CD25^+IL\text{-}10^+$ B cells in patients and beekeepers. The gating strategy for $CD73^-CD71^+CD25^+IL\text{-}10^+$ is shown in Fig. S4A. The frequencies of $CD73^-CD71^+CD25^+IL\text{-}10^+$ cells among $CD19^+$ cells increased significantly in the VIT group (1.2% ± 0.3 to 1.8% ± 0.4) (Fig 3A), and in the beekeeper group (1.51% ± 0.38 to 2.09% ± 0.51) (Fig 3B).

The frequencies of $CD73^-CD25^+CD71^+$ B cells did not change significantly during VIT in patients and after multiple bee stings during the season in beekeepers (Fig. S4B). We also investigated another subtype of IL-10-secreting Breg cells, $CD38^{hi}CD24^{hi}$ cells but there was no significant difference between the groups (Fig. S5).

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Frequency of IgG4-switched PLA-specific memory B cells increases after high-dose allergen exposure

The immunoglobulin heavy chain isotype is a key determinant of the effector functions executed by an antibody. We therefore investigated the isotype of the immunoglobulins produced by PLA-specific and PLA-non-specific class-switched memory B cells. The gating strategy for PLA-specific (PLA^+) and PLA-non-specific (PLA^-) immunoglobulins is shown in Fig.4A. The frequencies of PLA^+IgA^+ and PLA^+IgG1^+ B cells did not change significantly in response to VIT in allergic patients and after multiple bee stings in beekeepers (Fig 4B). Meanwhile, the frequencies of PLA^+IgG4^+B cells were significantly increased during VIT (7.20%±1.99 to 17.83%±3.27) (Fig 4B) and after multiple bee stings in beekeeper group (25.58%±3.68 to 39.20%±3.81) (Fig 4B). The data from Fig 4B are summarized in a stacked column graph in Fig 4C to illustrate the relative distribution of different immunoglobulin subclasses expressed by PLA-specific B cells. IgG4-switched PLA-non-specific B cells were detected only in very small frequencies (< 1%) and did not significantly change upon high-dose antigen exposure (Fig 4B-C).

We also measured the secretion of PLA-specific IgG1 and IgG4 antibodies from purified PLA-specific B cells. The amount of IgG4 secreted by purified PLA-specific B cells increased during VIT in patients as well as after multiple stings in beekeepers (Fig. S3C). PLA-specific IgG1 production by PLA-specific B cells did not change in response to VIT or multiple bee stings. Likewise, serum PLA-specific IgG4 increased during VIT and after multiple bee stings (Fig S6). Interestingly, the frequency of $IgG4^+$ PLA-specific B cells at baseline as well as after multiple bee stings was on average higher than in the VIT group before (three-fold) and during (two-fold) treatment (Fig4B-C). In contrast, the proportion of non-PLA-specific $IgG1^+$, $IgG4^+$ and IgA^+ B cells did not differ between the groups (Fig. 4B-C). Interestingly, $IgG4^+$ B cells comprised less than 1% of the PLA-non-specific B cells in all groups, indicating a relative dominance of IgG4-switched cells among allergen-specific B cells both in healthy and allergic individuals.

CCR5 expressing PLA-specific memory B cells are increased after high-dose allergen exposure

Next, we analyzed the expression of the chemokine receptors CXCR4, CXCR5, CCR5, CCR6 and CCR7 in PLA^+ and PLA^- class-switched memory B cells (Fig 5A). CCR5 expression increased significantly after high dose bee venom exposure in the both groups (Fig 5B). The expression level of CXCR4, CXCR5, CCR6 and CCR7 was not different

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between the groups. Chemokine receptor expression in PLA-non-specific class-switched memory B cells was not significantly different between before and after exposure in either group (Fig 5C).

DISCUSSION

This comparative analysis of peripheral B cells in allergic patients and healthy beekeepers before and after tolerance-induction by high-dose allergen exposure reveals intriguing similarities between B cell responses in the two groups. In this study we investigated the effect of high-dose allergen exposure on circulating naïve, memory and plasma cell fractions of allergen-specific B cells and compared these responses in two types of well-established allergen tolerance models, namely AIT and natural allergen exposure. As *in vivo* antigen-specific immune tolerance models, we used VIT-treated bee venom allergic patients, and compared this with non-allergic beekeepers, who were exposed to high doses of bee venom by multiple bee stings during the beekeeping season. We observed that allergen-specific B cell responses to high-dose venom exposure in patients and beekeepers showed striking similarities. In both groups we observed an increased frequency compared to baseline of IL-10-producing B_R1 cells, PLA-specific immunoglobulin heavy chain-switched memory B cells (particularly IgG4-switched cells), plasmablasts, and PLA-specific CCR5-expressing B cells in patients receiving VIT or after multiple bee stings during the beekeeping season in beekeepers.

Currently, several methods have been developed to examine antigen-specific B cells. Because of their low frequency, accurate detection and confirmation of specificity pose a challenge in the analysis of antigen-specific B cells. Fluorescent multimers have been used to detect peanut allergen-specific as well as tetanus toxoid-specific circulating B cells (25, 26). A dual-labeling strategy similar to what we used in this study was applied to investigate Ara h1 and Ara h2-specific B cells in peanut allergy (11). We demonstrate in this study that this method can be successfully applied to detect PLA-specific cells by measuring the production of PLA-specific antibodies from purified PLA-specific and non-specific B cells. We also found increased PLA-specific IgG4 in supernatants of purified PLA-specific B cells in patient samples during VIT and in beekeeper samples after multiple bee stings during the season, which corresponded to observed increases in with serum PLA-specific IgG4.

Plasmablasts are antibody-secreting B cells that are in the process of terminal differentiation towards plasma cells. We observed increased frequencies of circulating plasmablasts in patients during VIT and in beekeepers during the beekeeping season.

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Peanut-specific immunotherapy has been previously associated with an increased frequency of circulating allergen-specific class-switched memory B cells and plasmablasts (11, 26, 27). However, PLA-specific plasmablasts cells were rarely detected in our study. This could be due to downregulation of BCR expression during plasma cell differentiation, which impairs detection of antigen-specific BCRs. The frequency of class-switched PLA-specific memory B cells at baseline was higher in beekeepers than in bee venom allergic patients. This is most likely because beekeepers are chronically exposed to bee venom during multiple seasonal cycles, while allergic patients need to avoid contact with bees or wasps.

We subsequently analyzed the class of immunoglobulin heavy chain isotype that was expressed by PLA-specific B cells, and found that the proportion of PLA-specific IgG4-switched B cells was significantly higher in beekeepers than in bee venom allergic patients. After high-dose exposure to bee venom (VIT in patients or seasonal exposure in beekeepers), PLA-specific $IgG4^+$cells and serum PLA-specific IgG4 increased significantly in both groups. IgG4 is a blocking antibody isotype with anti-inflammatory potential. Induction of allergen-specific IgG4 was observed during the course of clinically successful in AIT (28, 29). The capacity of IgG4 antibodies to exchange Fab arms is a unique mechanism that leads to the formation of functionally monovalent antibodies that lack antigen-crosslinking capacity (9, 30-32). Moreover, allergen-specific IgG Abs can inhibit IgE-crosslinking on mast cells or basophils only when the IgG concentration and/or affinity are high in relation to the allergen dose (33). Allergen-specific IgG may inhibit the capture of IgE-allergen complexes by CD23 on B cells (34).

Circulating IgE-switched PLA-specific B cells could not be detected (data not shown). This may be due to very low frequencies of circulating IgE-switched memory B cells. It has been demonstrated that high affinity IgE antibodies develop primarily through sequential switching through an IgG intermediate (35). In humans, both direct and indirect class switch recombination to IgE occurs, and the relative contribution to low- and high affinity IgE antibody production remains to be elucidated (36). A recent study demonstrated that membrane-IgE-expressing B cells are self-restrained and rapidly undergo apoptosis, providing an explanation for the low frequencies of IgE^+ switched memory cells (37).

We demonstrated that B_R1 cells expanded in response to high-dose antigen exposure, both in patients receiving VIT and in beekeepers during the beekeeping season. We have previously shown that IL-10^+ B_R1 could suppress antigen-specific CD4+ T cell proliferation and up-regulate IgG4 production (24). Furthermore, regulatory B cells can induce Treg cells to infiltrate lungs, where they suppress allergic airway inflammation, while a deficiency of regulatory B cells increased allergic airway inflammation (38-42). Besides IL-

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10-producing B cells, TGF-β-producing B cells can suppress food allergy-induced intestinal inflammation through induction of Treg cells and suppression of Th2 responses (43).

Chemokines and their receptors regulate lymphocyte homing, and play a critical role in B cell migration. In this study, we investigated the expression of CXCR4, CXCR5, CCR5, CCR6 and CCR7 on PLA-specific B cells. While CXCR4, CXCR5, CCR6 and CCR7 expression was not affected by allergen exposure, CCR5 expression was significantly higher after high-dose bee venom exposure in patients and beekeepers. The natural ligands for CCR5 are macrophage inflammatory protein 1α (MIP1α/CCL3), MIP1β/CCL4, and regulated upon activation, normal T-cell expressed, and secreted (RANTES/CCL5). CCL5/RANTES serum concentration is elevated in insect venom allergic patients compared to healthy individuals, and decreases significantly during the course of allergen immunotherapy (44). CCR5 is involved in chemotaxis of immune cells to inflammatory sites in the periphery (16). An increasing body of evidence indicates that CCR5 expression by Treg cells is essential for their migration to inflammatory sites, and as such, is essential for Treg cells to exert their suppressive function *in vivo*. $CCR5^{-/-}$ Tregs, which were functional in suppressing T-cell proliferation *in vitro*, failed to prevent lethality in an acute graft-versus-host disease model (18, 19). $CD103^{+}$ tumor-derived Treg cells also required CCR5 expression in order to exert their *in vivo* suppressive function on anti-tumor responses (17). Another study demonstrated that, after nine months of house-dust mite immunotherapy, the proportions of $CCR5^{+}$ IL-10-producing T cells increased significantly, indicating that IL-10-producing $CD4^{+}$ T cells expand and acquire a peripheral tissue trafficking phenotype (18).

In conclusion, we demonstrate that allergen-specific B cell responses to chronic high-dose allergen exposure in allergic individuals and non-allergic beekeepers show remarkable similarities. These responses are characterized by expansion of PLA-specific class-switched memory B cells. These PLA-specific memory B cells are enriched for IL-10-producing B_R1 cells, and IgG4-switched memory cells, and thus adopt an anti-inflammatory profile. Allergen-specific IgG4 produced by these cells may prevent allergic responses and increased expression of surface CCR5 enables the migration of these allergen-specific B cells to peripheral sites inflammation, where they might function as suppressors of inflammatory responses. In order to prove that these phenotypical changes in the B cell compartment are required for tolerance induction to allergens, further studies are required in which responders and non-responders are compared. Because VIT is successful in > 95% of the patients it is challenging to obtain sufficient samples from non-responders (2). Therefore these findings should be investigated in larger cohorts of multiple types of AIT to assess their applicability as biomarkers of early and successful AIT response.

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AUTHOR CONTRIBUTIONS

TB and WV designed the study, performed, and analyzed experiments, and wrote the manuscript; NM, MM, MT, and AB recruited patients and collected patient material; MS and AE performed, and analyzed experiments; KR supported and made contributions to data interpretation. All authors revised and approved the final version of the manuscript.

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Figure legends

FIG 1. Increased frequency of PLA-specific class-switched memory B cells in response to VIT in allergic patients (**A**), and after multiple bee stings during the season in beekeepers (**B**). Cells were first gated as $CD19^{+}CD27^{+}IgM^{-}$. PLA-specific cells were identified as PLA-$AF647^{+}$ and PLA-$PC7^{+}$. (*Left*) Representative dot plots (means ± SEM), (*Right*) Cumulative data from VIT group (n = 14) and beekeeper group (n = 15). *** *P < 0.001.*

FIG 2. Increased frequency of plasmablasts during VIT in allergic patients (**A**), and after multiple bee stings during the season in beekeepers (**B**). (*Left*) Representative dot plots (means ± SEM). (*Right*) Frequencies of $CD27^{hi}CD38^{hi}$ plasmablasts (*right*) in VIT group (n = 14) and beekeeper group (n = 15). * *P < 0.05* *** *P < 0.001.*

FIG 3. Frequencies of B_R1 cells increase during bee venom immunotherapy in allergic patients and after multiple bee stings during the season in beekeepers. Frequencies of IL-

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10-producing B_R1 cells ($CD73^-CD71^+CD25^+IL\text{-}10^+$) in VIT group (n = 11) (**A**) and in beekeeper group (n = 15) (**B**). * $P < 0.05$, ** $P < 0.01$.

FIG 4. PLA-specific IgG4-switched B cells increase during VIT in allergic patients and after multiple bee stings during the season in beekeepers. PLA-specific cells were gated as $CD19^+IgM^-CD27^+$PLA-AF647$^+$PLA-PC7$^+$. (**A**) Representative staining of IgG1, IgG4 and IgA on PLA-specific (PLA$^+$) and PLA-non-specific (PLA$^-$) B cells. (**B**) Frequencies of IgA$^+$, IgG1$^+$ and IgG4$^+$ cells among PLA$^+$ and PLA$^-$ B cells in patients before and during VIT (*left*) and in beekeepers before and after multiple bee stings during the season (*right*). (**C**) Stacked column graphs represent immunoglobulin expression by PLA-specific and PLA-non-specific memory B cells. VIT group (n = 14) and beekeeper (BK) group (n = 15) ** $P < 0.01$, *** $P<0.001$.

FIG 5. CCR5 expression is upregulated on PLA-specific switched memory B cells in allergic patients during VIT and in beekeepers after multiple bee stings during the season. **A**, Representative histogram overlays of geometric mean fluorescence intensity (MFI) of CXCR4, CCR5, CCR6 and CCR7 staining. PLA-specific cells were gated as $CD19^+IgM^-CD27^+$PLA-AF647$^+$PLA-PC7$^+$ and PLA-non-specific cells were gated as $CD19^+IgM^-CD27^+$PLA-AF647$^-$PLA-PC7$^-$. Dotted line: isotype control (i.c.), light gray: before VIT or before bee stings (in beekeeper group), dark: after VIT (patients) or after multiple bee stings (beekeeper group). Cumulative data of chemokine receptor expression on PLA-specific switched memory B cells in allergic patients before and after VIT (n = 14) and beekeepers (n = 15) (**B**) and PLA-non-specific switched memory B cells (**C**). * $P < 0.05$, ** $P < 0.01$.

FIG S1. Gating strategy for PLA-specific cells in peripheral blood mononuclear cells. Lymphocytes were first gated from the forward and side scatter plot. After doublet discrimination, viable $CD19^+CD3^-CD14^-CD16^-$ cells were gated. Then $CD27^+IgM^-$ (class-switched memory B cells) and $CD27^+IgM^+$ (non-class-switched memory B cells) were gated. PLA-AF647$^+$ and PLA-PC7$^+$ cells were identified as PLA-specific cells. As a control for specific binding of antigen, cells were pre-incubated with non-labeled PLA to block subsequent binding of labeled PLA to the B cell receptor.

FIG S2. Detection of circulating PLA-specific $CD27^+IgM^+CD19^+$ non-class switched memory B cells in patients before and during VIT (**A**), and before and after multiple bee stings in beekeepers during the season (**B**). Representative dot plots of PLA-AF647 and PLA-PC7 staining (*left*) in beekeeper group and AIT group (means ± SEM). (*Right*) Frequencies of PLA$^+CD27^+IgM^+$ of B cells double stained with PLA-AF647 and PLA-PC7 in the VIT group (n = 14) and beekeeper group (n = 15). * $P < 0.05$.

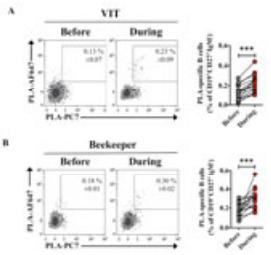
FIG S3. Immunoglobulin production by purified PLA-specific, and PLA-non-specific B cells. PLA-specific cells were gated as $CD19^+CD27^+IgM^-$ PLA-AF647$^+$PLA-PC7$^+$. Equal numbers of PLA$^+$ and PLA$^-$ cells were sorted before (white box) and during (grey box) VIT (n = 3) (**A**) and before and during the season in the beekeeper group (n = 5) (**B**). After three days of culture with CD40L and rIL-21, supernatants were collected and measured by ELISA for total IgG, PLA specific IgG1 and PLA specific IgG4. AU: Arbitrary units. **P < 0.01.

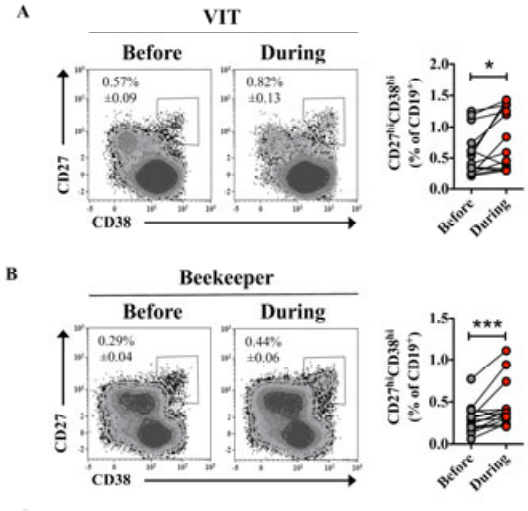
FIG S4. Gating of IL-10-producing BR1 cells **A,** Representative gating strategy for $CD73^-CD71^+CD25^+IL\text{-}10^+$ B cells. Samples were pregated on live $CD19^+$ cells. Percentages in dot plots indicate frequencies of gates cells among total CD19+ cells. **B**, Frequency of $CD73^-CD71^+CD25^+$ in VIT group (n = 11) and beekeeper group (n = 15).

FIG S5. Identification of $CD24^{hi}CD38^{hi}IL\text{-}10^+$ B cells. **A,** Representative gating strategy for $CD24^{hi}38^{hi}IL\text{-}10^+$ before and after bee venom VIT. Samples were pregated on live $CD19^+$ cells. Percentages in dot plots indicate frequencies of gates cells among total $CD19^+$ cells. Frequencies of $CD38^{hi}CD24^{hi}$ (**B**) and $CD38^{hi}CD24^{hi}IL\text{-}10^+$ (**C**) in VIT (n = 11) and beekeeper group (n = 15).

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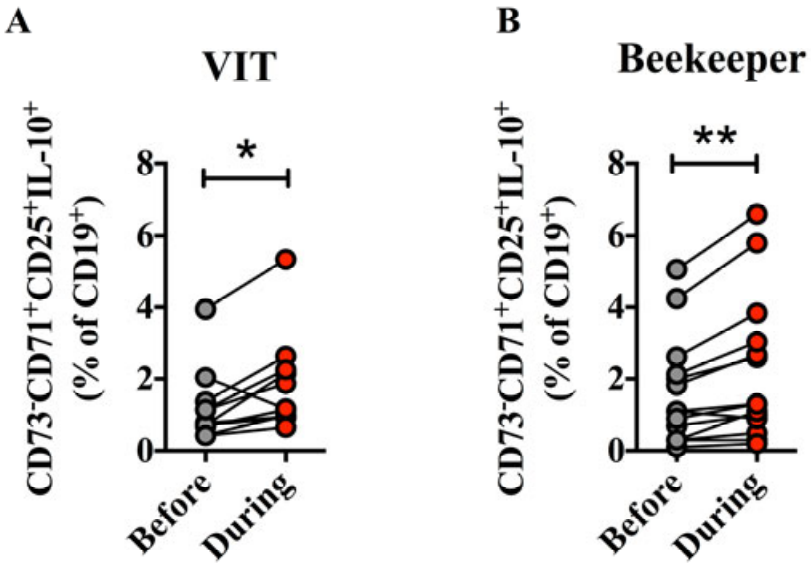
FIG S6. Serum PLA-specific IgG4 and IgE. Levels of PLA-specific IgE and IgG4 in serum were measured by ELISA from patient samples before and during VIT (n = 14) (**A**) and from beekeeper samples before and after multiple bee stings during the season (n = 14) (**B**). AU: arbitrary units. ** *P < 0.01.* *** *P < 0.001.*

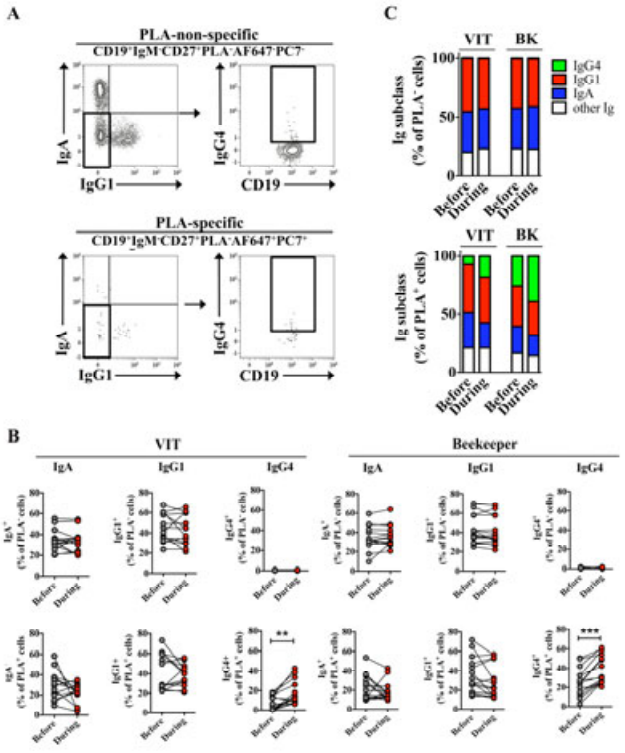




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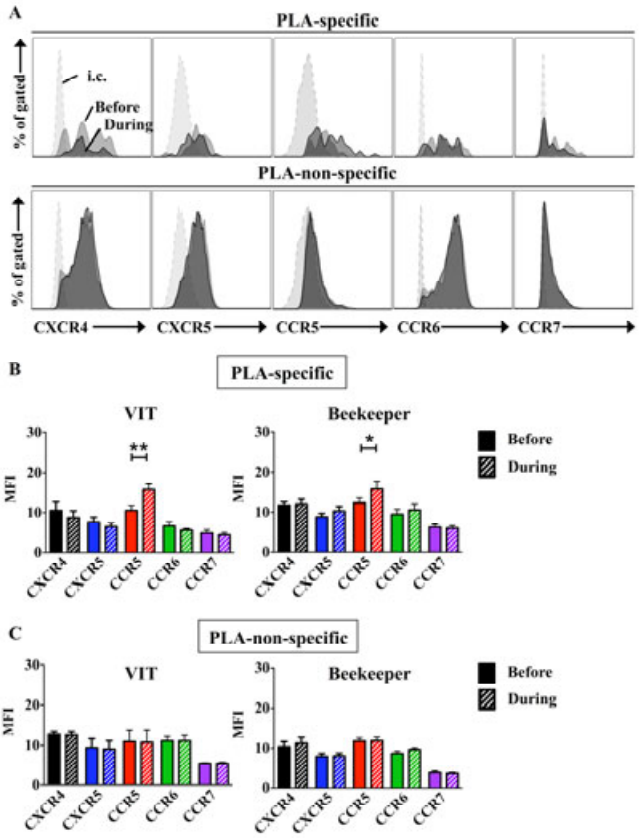
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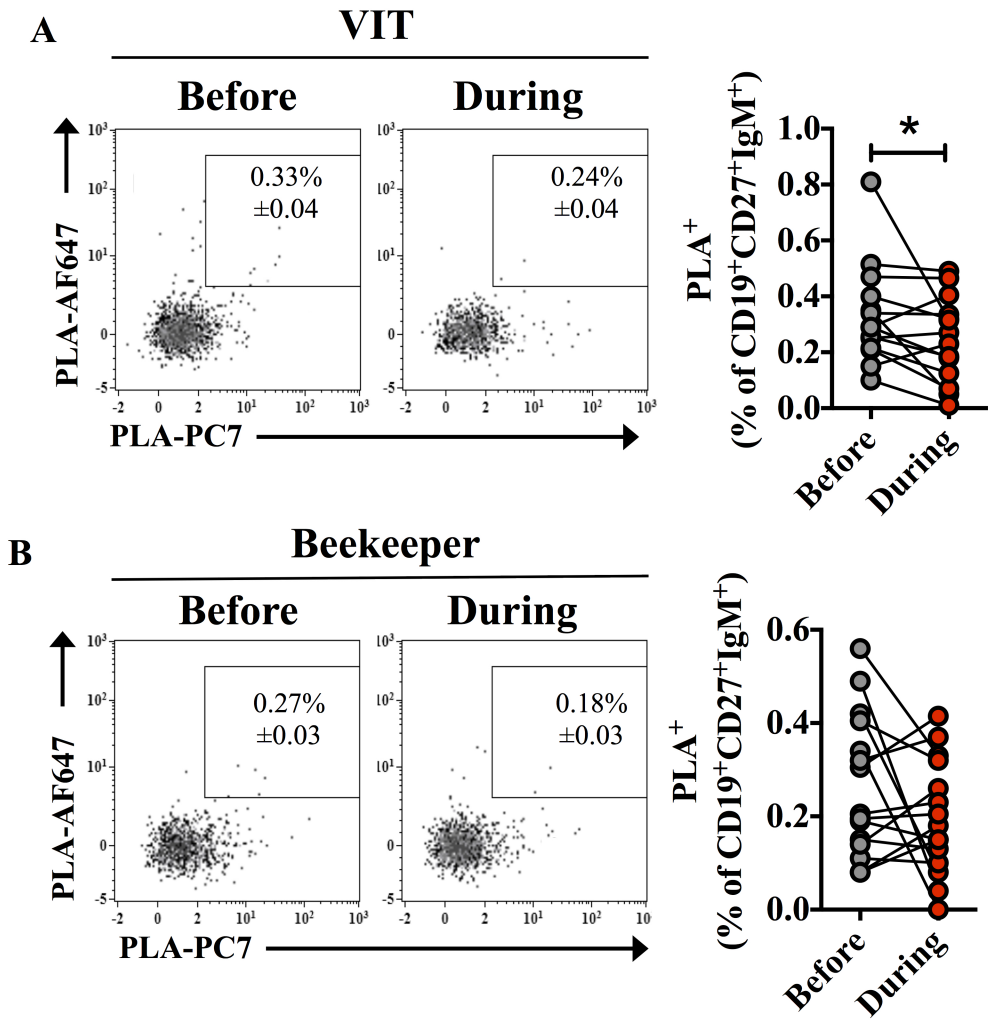


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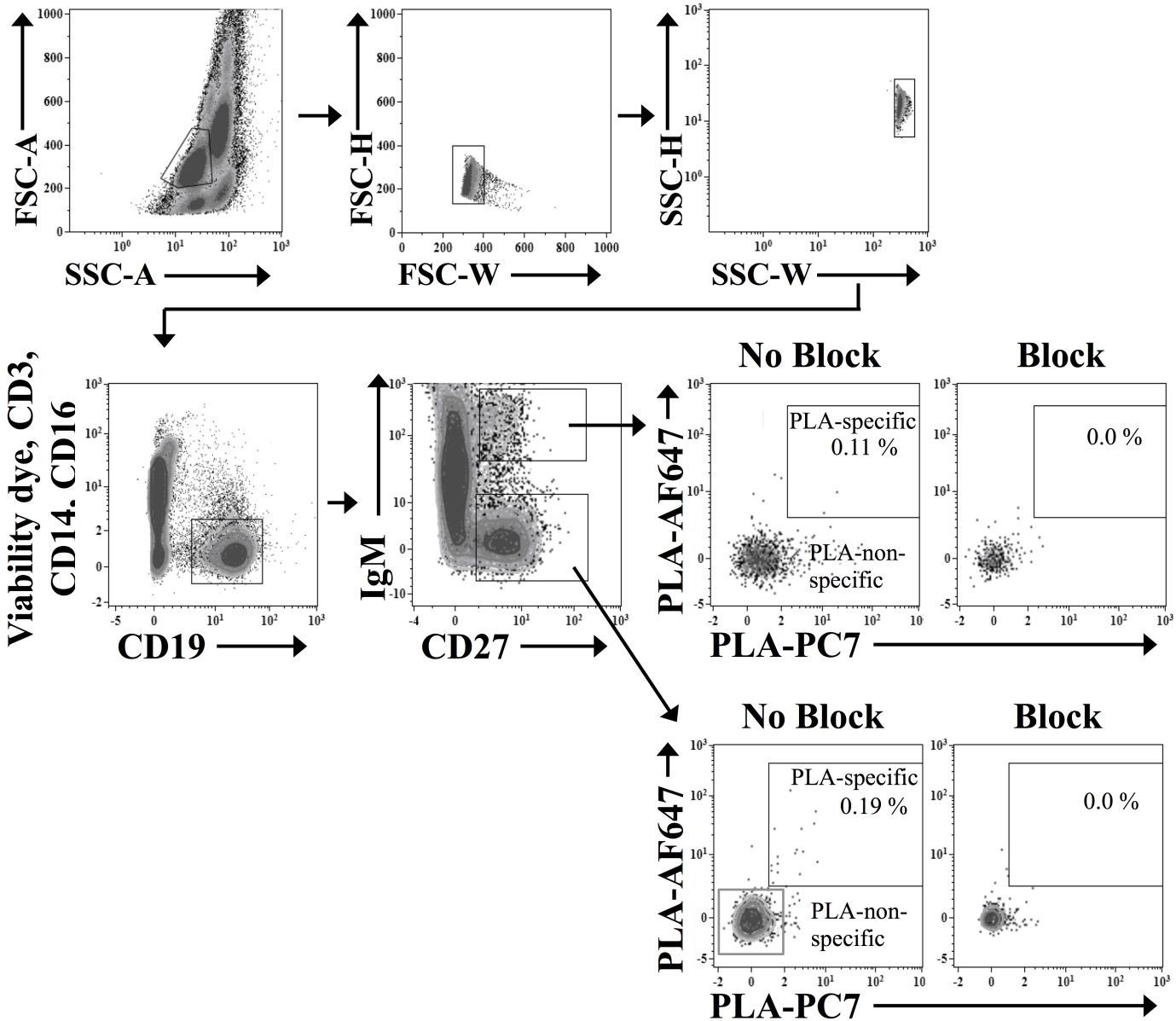


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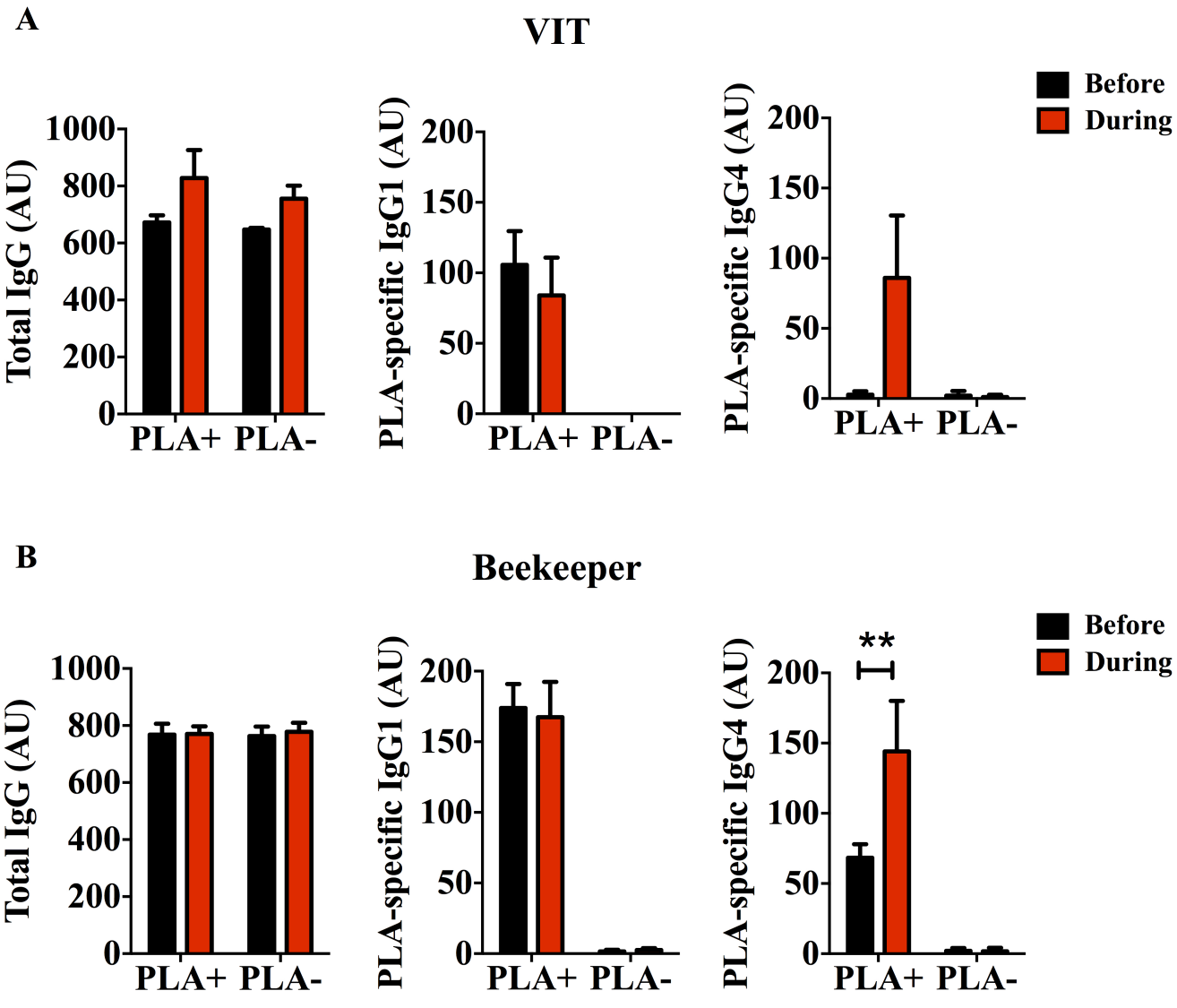


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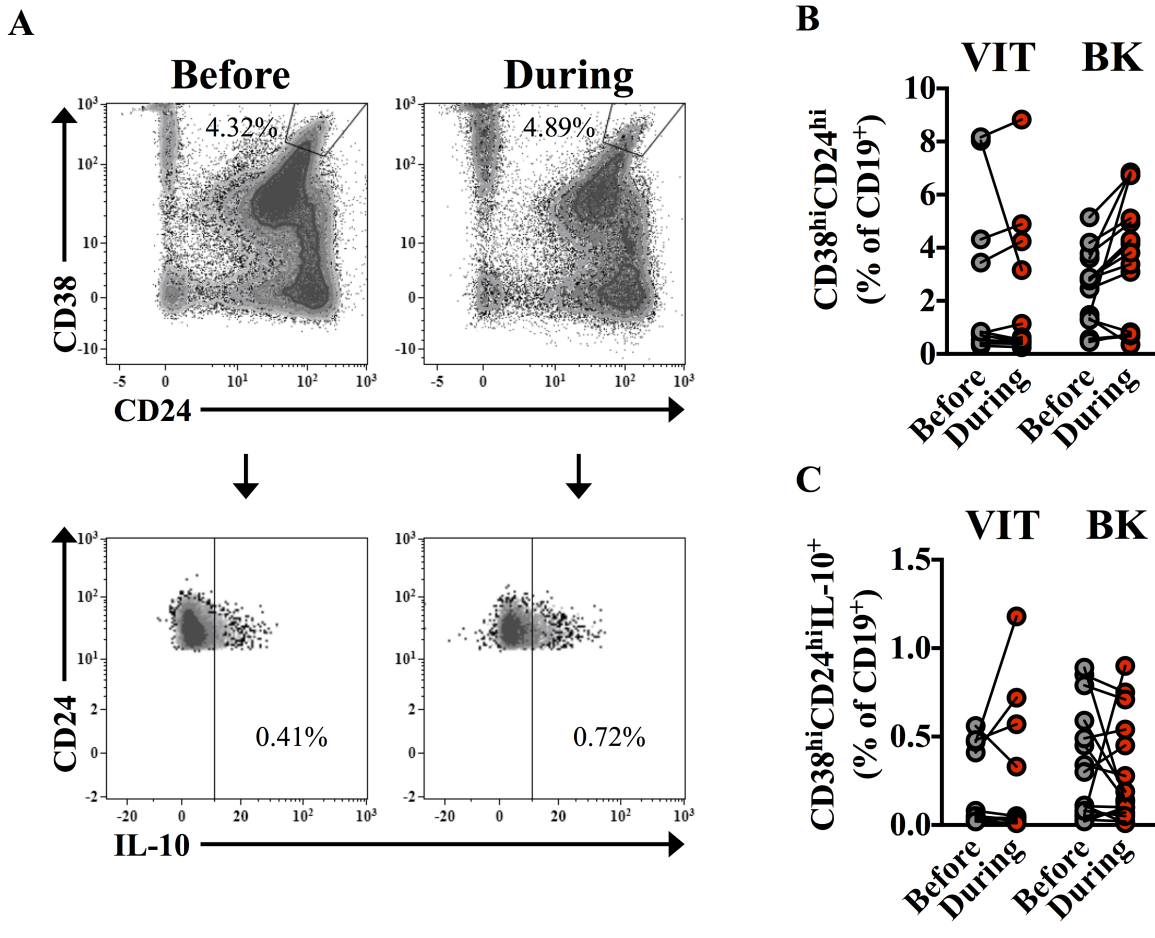
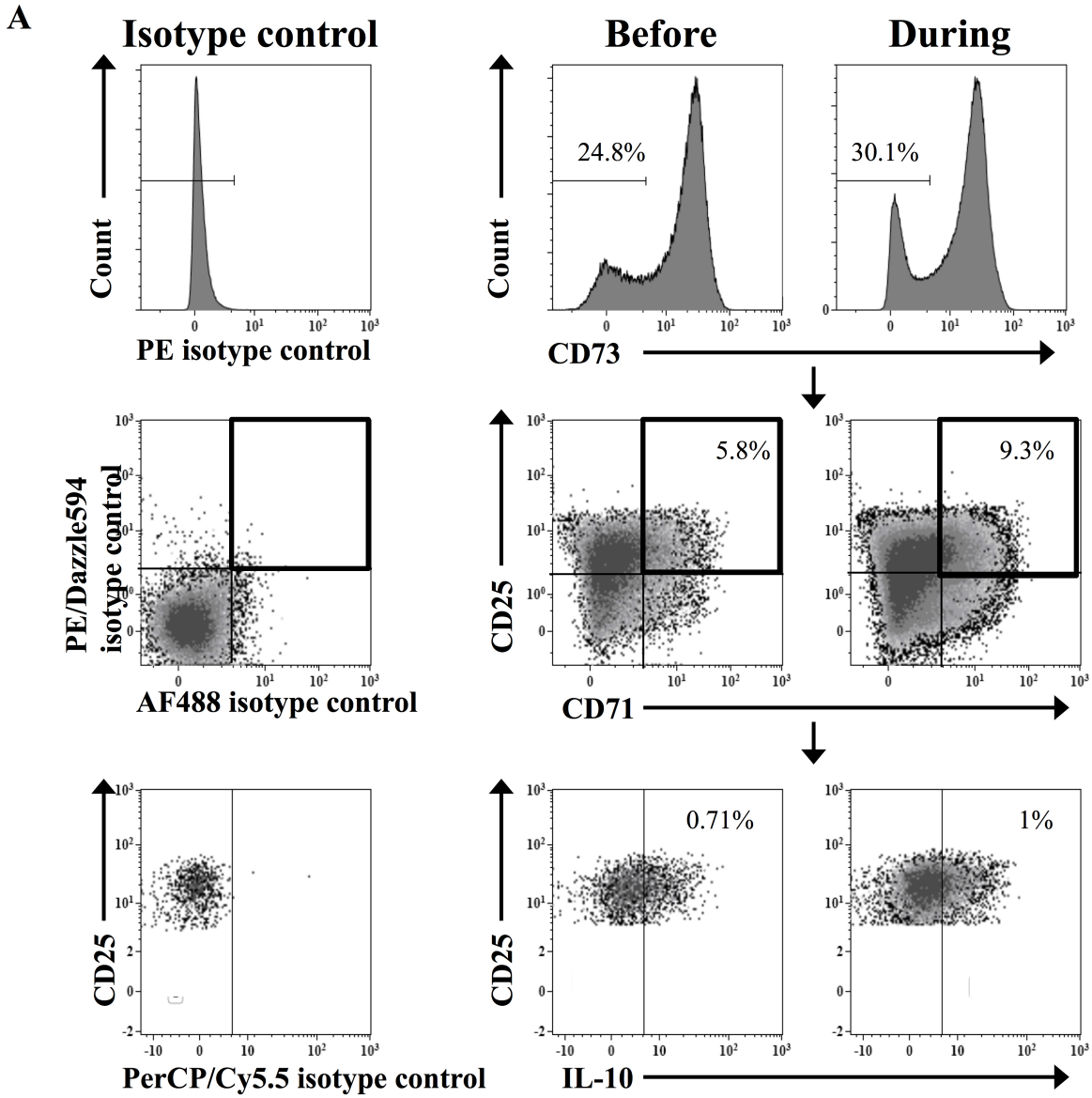


FIG S5. Identification of $CD24^{hi}CD38^{hi}IL\text{-}10^{+}$ B cells. **A,** Representative gating strategy for $CD24^{hi}38^{hi}IL\text{-}10^{+}$ before and after bee venom VIT. Samples were pregated on live $CD19^{+}$ cells. Percentages in dot plots indicate frequencies of gates cells among total $CD19^{+}$ cells. Frequencies of $CD38^{hi}CD24^{hi}$ (**B**) and $CD38^{hi}CD24^{hi}IL\text{-}10^{+}$ (**C**) in VIT (n = 11) and beekeeper group (n = 15).



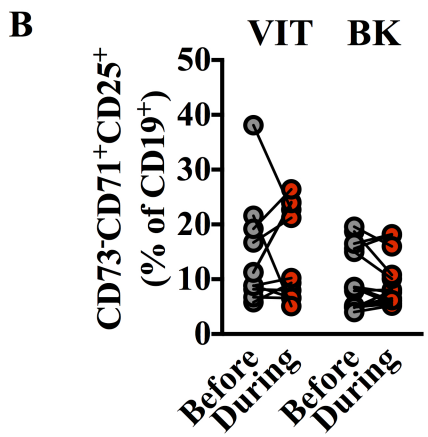


FIG S4. Gating of IL-10-producing BR1 cells **A,** Representative gating strategy for $CD73^{-}CD71^{+}CD25^{+}IL\text{-}10^{+}$ B cells. Samples were pregated on live $CD19^{+}$ cells. Percentages in dot plots indicate frequencies of gates cells among total CD19+ cells. **B**, Frequency of $CD73^{-}CD71^{+}CD25^{+}$ in VIT group (n = 11) and beekeeper group (n = 15).

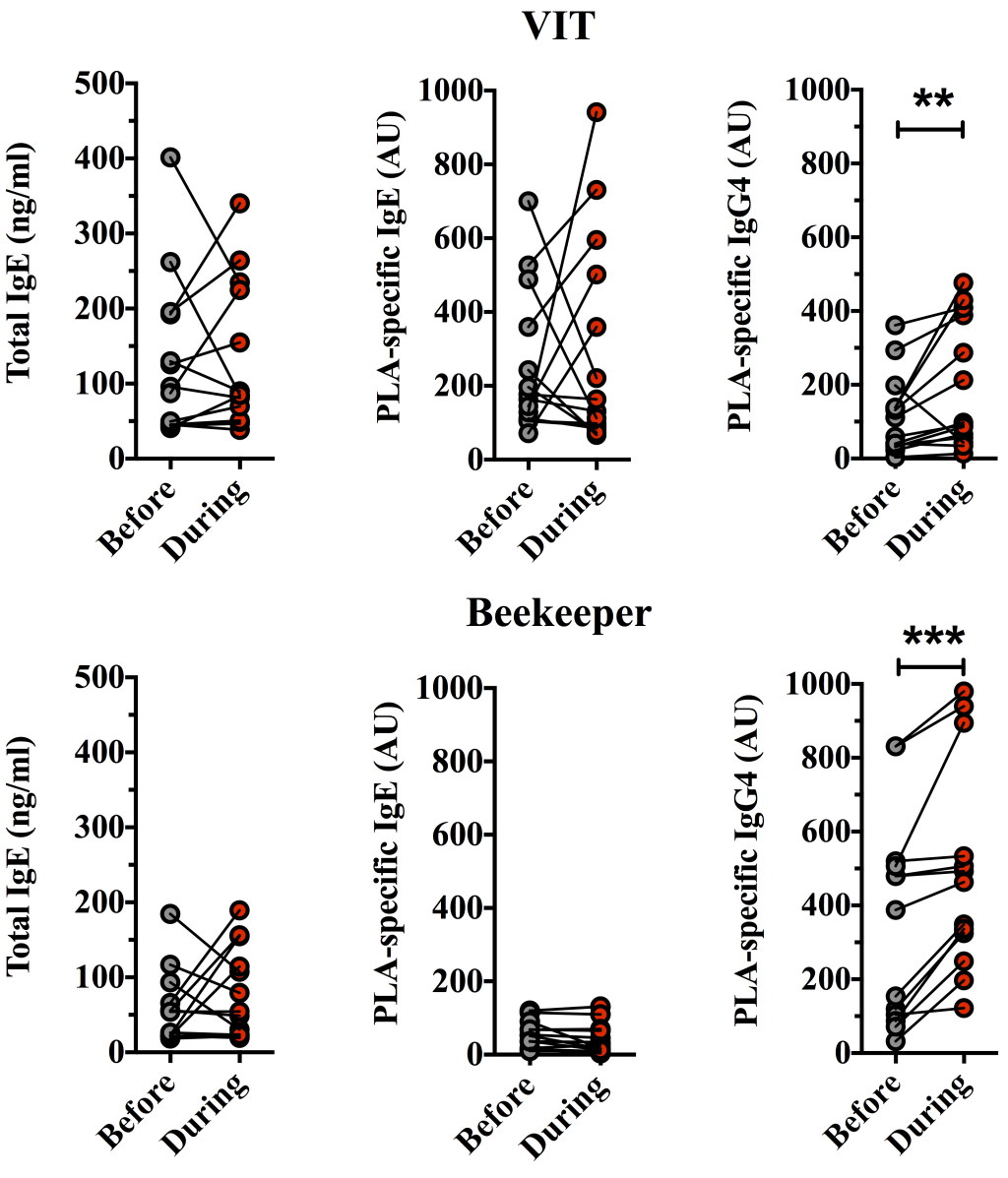


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Table S1. Venom-specific immunotherapy patient characteristics

Type of VIT	Age	Gender	Baseline specific IgE level (kU/L)	Baseline total IgE (ng/ml)	Character of reaction following exposure to honey bee sting (based on the Mueller scale)	Intradermal tests with honey bee venom (10^{-1})	Cumulative dose of Venomenhal Bee (Hal Allergy) received till the time of "during" analysis
Ultra-rush	31	Female	4.50	87.1	Anaphylactic SYS III	Positive 10/50	411.1 µg
Ultra-rush	48	Female	8.59	56.0	Anaphylactic SYSII/III	Positive	411.1 µg
Ultra-rush	40	Female	5.98	15.2	Anaphylactic SYS III	Positive 11x7/35	411.1 µg
Ultra-rush	33	Female	0.44	8.8	Anaphylactic SYS IV	Positive 12x6/30	411.1 µg
Ultra-rush	27	Male	5.18	111.0	Anaphylactic SYS III	Positive 11/25	311.1 µg
Ultra-rush	47	Male	15.00	ND	Anaphylactic SYS IV	Positive 12x7/35	411.1 µg
Ultra-rush	35	Female	11.10	49.8	Anaphylactic SYS III	Positive 8/40	211.1 µg
Ultra-rush modified	50	Male	18.30	316.0	Anaphylactic SYS IV	Positive 16/50	311.1 µg
Ultra-rush	35	Female	4.20	70.3	Anaphylactic SYS III	Positive 6/20	211.1 µg
Ultra-rush	59	Female	28.00	ND	Anaphylactic SYS III	Positive 10/50	711.1 µg
Ultra-rush modified	45	Female	>100	398.0	Anaphylactic SYS III	Positive 7x5/30	381,2 µg
Rush	35	Female	1.06	125.88	Asthma, urticaria	Positive	311 µg
Rush	25	Male	6.85	44.92	Asthma, anaphylactic	Positive	311 µg
Rush	51	Female	9.47	45.31	Asthma	Positive	311 µg

Table S2. Beekeeper characteristics

Age	Gender	Years active as beekeeper	Month of blood collection, before and during season	Bee stings received during season	Reaction after bee sting
57	Male	40	March and July	> 30	Local reaction, red+, swelling++, and pain+
42	Male	24	March and September	>150	Local reaction, red+, swelling+, and pain+++
48	Female	23	March and May	5	Local reaction, red+, swelling+, and pain+
32	Male	4	March and October	> 50	Local reaction, red++, swelling++ and pain+++
70	Female	50	March and October	>50	No reaction
60	Male	23	March and October	40	No reaction
51	Male	10	March and October	>150	Local reaction, red+, swelling+ and pain++
35	Female	5	March and May	5	Local reaction, no red, swelling++, and pain++
32	Male	4	March and May	25	Local reaction, red+, swelling+ and no pain
60	Female	43	March and August	40	Local reaction, no red, swelling+ and pain++
37	Male	7	March and September	37	Local reaction, red++, swelling++ and pain+++
58	Female	20	March and September	17	Local reaction, red+, no swelling and pain+
64	Male	31	March and October	74	Local reaction, red+, swelling++ and pain+
46	Female	5	April and October	13	Local reaction, no red, swelling++ and pain++
31	Female	4	April and October	65	Local reaction, red+, swelling+ and no pain

+, low; ++, mild; +++, moderate; ++++, severe and wheal > 10 cm diameter

None of the beekeepers showed systemic reaction and anaphylactic shock to bee stings.

All the beekeepers had > 100 bee stings in their life.

Table S3. Antibodies used for surface marker staining

Antigen	Label	Clone	Manufacturer
CD19	BV510	HIB19	Biolegend, San Diego, CA
CD27	AF700	M-T271	Biolegend, San Diego, CA
IgM	BV605	MHM-88	Biolegend, San Diego, CA
IgM	PerCP/Cy5.5	MHM-88	Biolegend, San Diego, CA
CD38	BV786	HIT2	BD Biosciences, Franklin Lakes, NJ
CD3	eFluro780	UCHT1	eBiosciences
CD14	APC/Cy7	HCD14	Biolegend, San Diego, CA
CD16	APC/Cy7	3G8	Biolegend, San Diego, CA
CD73	PE	AD2	Biolegend, San Diego, CA
CD25	PE	M-A251	Biolegend, San Diego, CA
CD71	PE/Cy5	M-A712	BD Biosciences, San Diego, CA
CD24	BV421	ML5	Biolegend, San Diego, CA
CCR7	AF488	G043H7	Biolegend, San Diego, CA
CXCR4	PE	12G5	Biolegend, San Diego, CA
CCR5	PE/Dazzle594	J418F1	Biolegend, San Diego, CA
CXCR5	PerCP/Cy5.5	J252D4	Biolegend, San Diego, CA
CCR6	BV421	G034E3	Biolegend, San Diego, CA
IgA	AF488	Goat-anti-hu IgA	Jackson Laboratories, West Grove, PA
IgG1	Dy405	HP6188	Sanquin, Amsterdam, Netherlands
IgG4	Biotin	RJ4	Alta Bioscience, Birmingham, UK
Anti-Biotin	PE	1D4-C5	Biolegend, San Diego, CA