Mechanisms of allergen-specific B cell tolerance in children with cow's milk-oral immunotherapy and natural outgrowth of milk allergy

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Abstract

Antigen-specific memory B cells play a key role in the induction of immune tolerance to food allergens and clinical healing. Here, we characterized the role of allergen-specific B cells in immune tolerance induced by oral allergen-specific immunotherapy (OIT) and natural tolerance that developed in children who spontaneously outgrew cow's milk allergy. Increased frequency of circulating milk allergen αS_1-casein -specific B cells was observed after OIT and natural tolerance (NT). Milk desensitized subjects showed partial acquisition of tolerance phenotypic features induced tolerance, suggesting that desensitization is an earlier stage of tolerance. Immunoregulatory genes such as *IL10RA* and *IGHG4* are significantly upregulated after OIT (desensitized and tolerance) versus NT. Secreted proteins from allergen-specific B cells revealed higher amounts of regulatory cytokines, IL-10 and TGF-β after OIT and NT. Taken together, allergen-specific B cells are essential elements in regulating food allergen tolerance in both OIT-received and naturally-resolved individuals.

Introduction

The prevalence of food allergy has been increasing in recent decades and affects approximately 10% of the world population[1, 2]. Cow's milk allergy (CMA) is a common disease in infants and children that shows a high rate of spontaneous resolution in early childhood until adolescence[3]. In some cases, CMA can result in anaphylactic reactions and has long-term implications for growth and nutrition. Avoidance of all cow's milk products in daily life is a standard physicians' recommendation to patients[4]. As an alternative to eliminating cow's milk from the patient's diet, oral allergen-specific immunotherapy (OIT) is an effective and curative treatment inducing clinical and immunologic tolerance to milk allergens in allergic patients[5, 6]. Understanding immune tolerance mechanisms to food allergens is crucial for further improving the existing treatments, and for the discovery of novel ways to prevent and treat food allergies[7, 8].

There are two major mechanisms which classify allergic reactions to cow's milk and other food allergens; immunoglobulin E (IgE)-mediated and non-IgE-mediated[9, 10]. The development of IgE-mediated CMA is regulated by B cells through the production of allergen-specific IgE antibodies[11]. Mechanisms driving B cells responses during allergy and development to tolerance remain to be elucidated. Hence, the investigation of B cell responses in food-allergic patients during OIT and in individuals who outgrow food allergy due to natural immune tolerance development may clarify mechanisms of induction and maintenance of food allergen tolerance.

Natural outgrowth of food allergies represents a valuable model to study mechanisms of immune tolerance to food allergens. So far, there have been very few well-designed studies regarding the natural development of tolerance to food allergens. Reported rates of resolution (natural outgrowth) vary widely, likely attributable to methodological differences and study populations. Some food allergens are difficult to avoid and fortunately have a generally high likelihood of natural outgrowth, such as cow's milk and

egg[2, 12, 13]. Safely loosening the diet to include milk and egg for children has important nutritional and quality-of-life benefits. It was demonstrated that tolerant children, who outgrew their allergy developed higher frequencies of circulating CD4+CD25+ Treg cells and decreased *in vitro* proliferative responses to bovine beta-lactoglobulin compared to children who maintained clinically active allergy[13]. Other food allergen sources, particularly peanuts and tree nuts, have a much lower spontaneous resolution rate and therefore require an efficient OIT approach[2, 12].

Molecular mechanisms of spontaneous outgrowth of food allergies have not been studied in detail and there is no report on the role of allergen-specific B cells in natural tolerance. Hoh *et al.* investigated B cell responses in peanut allergy and demonstrated that local class-switch recombination (CSR) to IgE in the gut directly has a major impact on the development of food allergen-specific IgE antibodies[14]. Circulating IgE+ B cells display mostly immature plasmablast phenotype[15]. Increased numbers of circulating IgE+ memory B cells and IgE+ plasmablasts are correlated with the presence of food allergy and may contribute to pathogenesis[16].

Here, we have performed an in-depth characterization of B cells specific for αS_1-casein, the major allergen in cow's milk, to analyse B cell changes related to the development of tolerance to cow's milk allergy. We identify an increased frequency of allergen-specific B cells in OIT induced-cow's milk tolerance and natural outgrowth-related immune tolerant individuals. Allergen-specific B cells predominantly express IgG4 after OIT and natural tolerance, with smaller numbers expressing IgG1 or IgE. Gene expression signatures associated with allergen-specific B cells were mostly downregulated after OIT, showing that allergen-specific B cells decrease their expression of 1456 genes and downregulate activation and proinflammatory gene expressions profile. Notably, secreted protein profiles of specific B cells were similar after OIT and NT, suggesting common but not identical mechanisms of food allergen tolerance.

Results

Increased frequency of αS_1-casein-specific B cells in OIT-induced tolerance and natural tolerance

The frequencies of αS_1-casein-specific B cells (CD19+IgM-CD27+) in circulation was calculated by direct analyses of the cells without culturing or other ex vivo manipulations. The details of the gating strategy are shown in supplementary Fig. 1. αS_1-casein-specific B cells were found at low frequencies of 0.01–0.5% in three main representative groups including healthy control (HC), allergic groups (before OIT, during OIT, and after OIT), and natural tolerance (NT) (Fig. 1A). Mean frequencies ± standard deviation was 0.03±0.03 in HC, 0.16±0.06 before OIT, 0.29±0.13 in during OIT, 0.28±0.14 in after OIT, and 0.37±0.15 in NT (Fig. 1B). A significant increase in allergen-specific B cell frequency was observed in the course of OIT. The highest frequency of allergen-specific B cell was in the NT group. While HC exhibited the lowest frequency of αS_1-casein-specific B cells. These data demonstrate that allergen-specific B cell frequencies are increased during OIT and NT development.

αS $_1$ -casein-specific B cells produce predominantly IgG4 in OIT and natural tolerance

To verify allergen-specificity and isotype expression of αS_1-casein-specific B cells, we detected specific Abs including sIgG, sIgG1, sIgG2, sIgG3, sIgG4, sIgA1, sIgA2, and sIgE in supernatants from $5x10^5$ immortalized αS_1-casein-specific and non-specific B cells with 0.6 μM αS_1-casein allergen stimulation for 72 hours.

From the ELISA results, αS_1-casein-specific IgG was 0.39±0.16 in HC+, 0.5±0.08 in before OIT+, 0.64±0.11 in after OIT+, and 0.84±0.07 in NT+, αS_1-casein-specific IgG1 was 0.13±0.02 in HC+, 0.24±0.07 in before OIT+, 0.16±0.01 in after OIT+, and 0.3±0.04 in NT+. αS_1-casein-specific IgG4 also changed as 0.13±0.03 in HC+, 0.17±0.05 in before OIT+, 0.41±0.06 in after OIT+, and 0.53±0.09 in NT+. αS_1-casein-specific IgE was 0.03±0.01 in HC+, 0.26±0.05 in before OIT+, 0.22±0.07 in after OIT+, and 0.12±0.02 in NT+. In contrast, sIgG2, sIgG3, sIgA1, and sIgA2 were undetectable or very low in B cell supernatants (data not shown). The production of αS_1-casein-sIgG, sIgG1, sIgG4, and sIgE was significantly higher in specific B cells from HC, before OIT, after OIT, and in NT compared to the non-specific B cells (Fig. 2A). These results confirm that the sorted single allergen-specific B cell populations were highly pure and expanded. Importantly, we observed that αS_1-casein-specific B cells from healthy individuals did not produce sIgE and rather showed sIgG1 and sIgG4 production. In contrast, food-allergic individuals had relatively high sIgE production together with sIgG1 and relatively low sIgG4 isotype Abs. The levels of these Abs showed a very significant change after OIT. sIgG4 became the major Ab isotype with quite low sIgG1 without showing any change in sIgE Abs. For comparison, NT demonstrated a high sIgG4 Ab response with moderately low sIgG1 and sIgE. To further demonstrate this change in the prevailing specific Ab isotype, the sIgE/sIgG4 ratio was calculated in all groups. In the NT and HC (0.24±0.05 and 0.48±0.56, respectively), the sIgE/sIgG4 ratios were significantly lower compared to before OIT (1.65±0.7) and after OIT (0.55±0.2, respectively). Allergic patients before OIT had the highest IgE/IgG4 ratio, whereas the natural tolerance group had the lowest. (Fig. 2B).

Downregulation of αS $_1$ -casein-specific B cells in OIT-induced desensitization and significant silencing in tolerance

To further delve into the *in vivo* functional changes in αS_1-casein-specific B cells that could be related to immune tolerance, they were freshly isolated and immediately analysed using the ultra-low input RNA-sequencing method without any cell culturing. Differentially expressed genes (DEGs) were identified in the gene expression profile of αS_1-casein-specific and non-specific B cells from before and after OIT with 2 outcomes: desensitized and tolerance (Fig. 3A). OIT outcomes were defined based on allergic reaction results from cow's milk extract re-challenge at six weeks after the immunotherapy. Patients who showed any reaction more than 32.7 mg dose during the challenge, were classified as desensitized. Patients who tolerated the 8,000-12,000 mg dose without any clinical reactions were identified as tolerance patients. Immunotherapy-induced genes were defined as DEGs in comparisons of pre-OIT and after OIT where the p-value was lower than 0.05. RNA-seq data from participants with desensitized and tolerance outcomes

after OIT (4 desensitized and 5 tolerance) were analysed. Fig. 3A shows the top 200 most significantly downregulated genes in αS_1-casein-specific B cells after OIT compared to before OIT. There was a clear difference in gene expressions between desensitized and tolerance groups. The desensitized group showed down-regulation of certain clusters of genes compared to the pre-OIT state, but the tolerance group showed a markedly greater decrease in expression of these genes, approaching a complete lack of expression in some cases. In contrast, there was no change in expression of these genes pre- and post-OIT in non-specific B cells representing the switched memory cells. Biological pathways potentially affected by DEGs were further analysed using the Metacore and Enrichr programs. Ten B cell-related pathways including immune response-mediated by circulating Igs, regulation of B cell activation, B cell receptor signalling, cytokine-mediated signalling, antigen receptor-mediated signalling, cellular response to cytokine stimulus, inflammatory response, regulation of immune response, B cell activation involved in immune response, and chemokine-mediated signalling were identified (Fig. 3B, supplementary table 1). A volcano plot from αS_1-casein-specific B cells after OIT versus before OIT showed 1,456 significantly downregulated genes and 40 significantly upregulated genes (Fig. 3C).

To characterize the genes that were upregulated after OIT by their roles and functions from the literatures search, we compared B cell-related DEGs between desensitized and pre-OIT samples in Fig. 3D. B cell antigen-binding genes (*TAP1, ITGA4, EP300*) were significantly increased in the desensitized patients compared to before OIT. B cell cytokines and their receptors; *IL12RB2* and *TNFRSF10D* were completely downregulated in the desensitized group. B cell activation showed higher expression of *TLR4* in the allergic group that decreased during desensitization, but *FOXP1* and *JAK3* were increased in the desensitized group. B cell chemokine genes including *PLAU, CX3CR1, CCL3L3, XCL2* showed a significant decrease in the desensitized group. In contrast, *CCR6*, a gut homing chemokine receptor was highly expressed in desensitized patients. Breg cell-related genes including *AHR, GZMB, GZMH* decreased during desensitization, but *IL10RA* and *TGFB3* showed a very significant increase. BCR signalling (*PIK3CA, PIK3CD, PIK3CG*), B cell differentiation (*BCL11A, CARD11, DOCK11, MALT1*), as well as B cell immunoglobulin genes (*IGHG2*) showed a significant increase after desensitization.

In the comparisons between the allergic and tolerance groups (Fig. 3E), we found that B cell cytokines/cytokine receptors (*IL1RAP, IL12RB2, IL18R1*) and B cell chemotaxis (*PLXNA1, PLXNA2, XCL1, XCL2*) showed a decrease in the tolerance group after OIT. Interestingly, *IGHG4* which has been repeatedly reported in other allergen tolerance studies showed a significant increase in the tolerance group[17]. Other Breg cell-related genes (*TGFB3, ACVR1C, ACVR2B*) had low expression. In contrast, B cell immunoglobulins constant regions (*IGHG1, IGHA1, IGHA2, IGHD*) are highly increased in tolerance subjects. Notably, the *IGHD* gene encodes the heavy chain of IgD, which was previously reported to increase in serum from cow's milk-allergy resolved cohort[18].

These data demonstrate clear differences in the gene expression signatures of desensitized, and tolerance patient's allergen-specific B cells compared to the pre-OIT state. A large number of B cell activation, cytokine, chemotaxis and differentiation-related genes are downregulated after OIT. Our

findings suggest that specific B cells under the influence of OIT, downregulate the cellular activation gene machinery in desensitized patients and mostly shut down during the tolerance state.

Differences in αS $_1$ -casein-specific B cells between OIT-induced desensitization and tolerance

We further analysed the overall gene data sets with a principal component analysis (PCA). This PCA plot demonstrates the top 200 DEGs that are separated into 4 different groups; before OIT, desensitized, tolerance, and NT group (Fig. 4A). DEGs characteristic of each group and clinical outcome were identified. B cells from patients with desensitization outcomes had intermediate results in comparison to pre-OIT and tolerance patient B cells. We evaluated the distinctive OIT-induced genes in desensitized and tolerance groups. Fig. 4B and 4C show that B cell antigen-binding (*TAP1*), BCR signalling (*PIK3CA, PIK3CD, PRKCB*), B cell activation (*RIF1, RNF168, SWAP70, FOXP1, JAK3*), B cell chemotaxis (*CCR6*), B cell cytokines/cytokine receptors (*IL21R*), B cell differentiation (*BCL11A, CARD11, DOCK11, MALT1*), and Breg cell-related genes (*IL10RA*) were further decreased in tolerance patients compared to desensitized patients. In contrast, *TLR4* showed a significant increase after tolerance. Moreover, B cell immunoglobulins (*IGHG1, IGHA1, IGHA2)* were highly expressed in tolerance patients, suggesting B cell class switch recombination in relation to antigen-specificity and plasma cell differentiation in tolerance memory B cells.

Distinct gene signatures of αS $_1$ -casein-specific B cells between outgrowth from milk allergy by natural tolerance compared to milk allergic children

This study also enabled us to delve into mechanisms of natural tolerance. Accordingly, we compared the differences in gene expression signatures between αS_1-casein-specific B cells from food allergic individuals versus NT in circulating antigen-specific B cells representing the *in vivo* condition. A heatmap of DEGs highlights differences in the gene expression profile of αS_1-casein-specific B cells before OIT compared to NT (Fig. 5A). B cell-related pathways were analysed and shown in Fig. 5B and supplementary table 2. A volcano plot from αS_1-casein-specific B cells before OIT versus NT show 1,693 significantly downregulated genes and 704 upregulated genes (Fig. 5C). Although regulation of gene expression suggested less B cell activation and induced allergen tolerance during OIT, we did not see the same type of changes in NT. These findings suggest that different types of mechanisms may play a role in NT. Interestingly, the changes in NT were relatively heterogeneous compared to the gene expression of specific B cells in allergic individuals. Allergic children showed a high number of significantly increased B cell activation-related genes. Many of them were significantly downregulated in specific B cells upon NT (Fig. 5D). A significant number of antigen-specific B cell activation molecules was downregulated in NT. An exciting finding was the expression of type 2 cytokine receptor genes (*IL4R, IL13RA*) was significantly lower in NT allergen-specific B cells compared to those of allergic children. Significantly lower expression of antigen-presentation-related costimulatory receptors (*CD40, TAP1)*, B cell activation (*BATF, HRH2, TLR2, ADA*), cytokines or cytokine receptors (*IL7R, IL12RB1, IL12RB2, IL18R1, IL23A*), chemotaxis (*PLAU, CXCR3, XCL1, XCL2, CCL3L3, CCL4L2, CCRL2*), Breg-related genes (*GZMB, GZMH, ACVR1B, TGFB3*), BCR signalling genes (*PIK3R3*), and B cell differentiation (*HMGB3*) was also seen in NT.

NT was associated with a group of upregulated genes in B cells, such as *NOTCH2, FOXP1, TLR1, TLR6, TLR9*, cytokine/cytokine receptors (*IL21R, IL2RG*) that are important in clonal expansion of established tolerance related clones, and chemokine genes (*TNFRSF1B)*, BCR signalling (*CD22*, *PIK3CD)*, B cell differentiation (*BCL6, CARD11)* and immunoglobulin genes (*IGHD)*. Several of these genes display a direct or indirect relationship to immune tolerance and plasma cell differentiation. These data indicate a complementary shutdown mechanism by allergen-specific B cells in natural tolerance and OIT-induced tolerance, however, regarding their immune-tolerance-related properties and kinetics, DEGs do not exactly span the same molecules in their roles and biological functions.

Comparison of gene signatures of αS $_1$ -casein-specific B cells in OIT-induced tolerance and natural tolerance

To compare gene signatures between αS_1-casein-specific B cells from tolerance-induced after OIT versus NT, we first generated a heatmap of most significant DEGs (Fig. 6A). B cell-related pathways were analysed and shown in Fig. 6B and supplementary table 3, which showed similar pathways in induced tolerance and NT. A volcano plot from αS_1-casein-specific B cells after OIT versus NT showed 1,303 significantly downregulated and 552 upregulated genes (Fig. 6C). 358 genes showed similar changes in OIT and NT patient B cells compared to pre-OIT samples, indicating some similar mechanisms in tolerance-induction by OIT and NT (Fig. 6D).

We observed that B cell activation (*STAT6, FOXP1, NOTCH2, TLR9*), B cell cytokine/cytokine receptors (*IL2RG, IL7*), Breg cell-related genes (*AHR*), B cell differentiation (*BCL6, CARD11*, *DOCK11*), BCR signalling (*CD22, PIK3CA*), and immunoglobulin (*IGHD*) are high in NT group. In contrast, OIT induced tolerance displayed higher levels of gene expression in antigen-binding (*CD40, TAP1*), cytokine/cytokine receptors (*IL23A, IL21R*), Breg cell-related genes (*IL10RA*), B cell differentiation (*CD79A, CD79B*), and immunoglobulin (*IGHG1, IGHG4, IGHA1, IGHA2*) (Fig. 6E).

Gene signatures from immunotherapy-induced tolerance in specific B cells show marked changes compared to the pre-OIT state. However, these changes did not fully overlap with those seen in patients who achieved natural tolerance. Most importantly, *IL10RA* and *IGHG4* genes encoding cytokine IL-10 and IgG4 were expressed more in the induced-tolerance state.

Secreted proteins from αS $_1$ -casein-specific B cells in children with cow's milk induced tolerance and natural tolerance display similarity

We further analysed the secreted proteins from purified αS_1-casein-specific B cells, that were immortalised and expanded. Proximity extension assay (PEA) technology was used to measure 92 proteins in a multiplex assay (inflammation panel). Heatmap of differentially expressed proteins showed significantly secreted proteins from specific B cells before OIT, after OIT (tolerance), and NT (Fig. 7A). The GO Biological processes pathways were analysed and shown in Fig. 7B and supplementary table 4. The

23 most significant differentially expressed proteins are shown in Fig. 7C and the remaining of non-significant plots are included in supplementary Fig. 3.

As a major finding, we observed that the secretion of immunoregulatory cytokines IL-10 and latency activated protein (LAP) of TGF-beta (TGF-β), that belong to B cell activation pathway, were increased in the supernatants of specific B cells from the NT group. Similarly, several proteins including adenosine deaminase (ADA), caspase-8 (CASP-8), IL-7, were also increased in NT compared to before OIT. From chemotaxis pathway; urokinase-type plasminogen activator (uPA), cytokine-mediated signalling pathway; macrophage colony-stimulating factor 1 (CSF-1), Fms-related tyrosine kinase 3 ligand (Flt3L), tumor necrosis factor receptor superfamily member 9 (TNFRSF9), and cell-surface receptor signalling pathway; 4E-binding protein 1 (4E-BP1), STAM-binding protein (STAMBP), metalloproteinase-1 (MMP-1), axin-1 (AXIN1), osteoprotegerin (OPG) were also increased.

Interestingly, 13 proteins in these four different pathways such as LAP TGF-β, TNFRSF9, AXIN1, uPA, STAMBP, C-X-C Motif Chemokine Ligand 10 (CXCL10), C-C Motif Chemokine Ligand 20 (CCL20), vascular endothelial growth factor A (VEGFA), and IL-6 were significantly increased in after OIT-induced tolerance when compared to before OIT. In contrast, monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 4 (MCP-4), matrix metalloproteinase-10 (MMP-10), and Artemin (ARTN) were highly expressed in OIT-induced tolerance group. In addition, 7 proteins, TNFRSF9, ADA, and CSF-1 showed higher expression in NT compared to OIT-induced tolerance, but AXIN1, MMP-10, ARTN, and IL-6 showed lower expression.

In conclusion, the majority of secreted protein molecules from allergen-specific B cell cultures that display immune-regulatory and anti-inflammatory properties are highly secreted in natural tolerance and induced-tolerance as compared to allergic conditions.

Discussion

The present study provides an in-depth investigation of allergen-specific B cells from children with cow's milk-induced tolerance and NT. Although cow's milk is one of the most important and basic nutrients introduced early in life in the diet, it can induce IgE-associated food allergy that causes severe allergic manifestations in the gut, skin, and even in the respiratory tract and may lead to life-threatening anaphylactic shock. The major cow's milk allergens belong to the casein group of proteins (αS_1, αS_2-, β-, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin)[19, 20]. We focused our investigation on a direct *in vivo* analysis of freshly isolated αS_1-casein allergen-specific B cells not manipulated by cell culture or other procedures. Additional experiments with these low-frequencies of specific B cells included immortalization of them to confirm their antigenic specificity, and measurement of cytokine expression profiles.

Our approach in the identification of αS_1-casein-specific B cells was performed with a double-allergen labelling method that was established in several studies including the identification of peanut-specific B

cells (Ara h1 and Ara h2)[21], bee venom-specific B cells (PLA)[22], and house dust mite-specific B cells (Der p 1)[23]. We found significantly increased percentage of circulating allergen-specific B cells in allergic individuals, which continued to increase during, and after OIT. Increased frequencies of allergen-specific B cells were demonstrated in a previous study in house dust mite allergic patients after 2 years of immunotherapy[23]. We observed very low frequencies of allergen-specific B cells in HC. Interestingly, the highest frequencies of allergen-specific B cells were observed in NT, suggesting the *in vivo* expansion of functional allergen-specific B cells during spontaneous outgrowth of allergies probably due to continuous exposure afterward.

Indeed, we confirmed the specificity of sorted B cells for αS_1-casein, and our results exhibited the higher levels of αS_1-casein-sIgG, sIgG1, sIgG4, and sIgE in supernatants of specific B cells. In addition, we detected sIgE in the milk allergic and OIT-receiving group, but not in HC and it was at the low levels in NT. sIgG4 isotype was predominantly high after OIT and in the NT group. It has been demonstrated in other allergens, such as bee venom, egg, and house dust mites that exposure to allergens induces an immunologic response that specifically enhances IgG4 levels due to continuous allergen exposure[23, 24, 25, 26]. The allergic group showed a significant decrease in sIgE/sIgG4 ratios after OIT, and even lower sIgE/sIgG4 ratios were observed in the NT group and HC. Therefore, low sIgE and sIgG4 Ab levels in cow's milk allergic children may be a predictive marker in the achievement of natural tolerance and successful response to OIT[27].

There was a clear difference between tolerance and desensitized B cells suggesting that desensitization is an intermediate step on the way to tolerance development. In both the tolerance and desensitized groups, antigen binding, cytokines and receptors, B cell activation, chemotaxis, BCR signalling, B cell differentiation, and B cell Ig heavy chain genes were altered. There was an increase of Breg cell-related genes, BCR signalling and differentiation. Within these most significantly increased genes, *IL10RA*, a receptor for the binding of suppressor cytokine IL-10 and *TGFB3* a regulatory cytokine for antigen/allergen tolerance were highly expressed in desensitized OIT patients. Besides, the expression of *CCR6*, a gut homing chemokine[28], was also higher in desensitized individuals, suggesting the OIT-induced-tolerance state might occur in the gut.

Changes in the expression of B cell IGH genes showed interesting findings. While in the desensitized patients, only an increase in IGHG2 was observed, in tolerance subjects, we saw a significant increase in genes encoding the heavy chains for IgG4, IgG1, IgA1, IgA2, and IgD. An increase in all of these specific antibody isotypes has been linked to tolerance development in serum in AITs with different allergens[27, 29, 30]. Among all immunoglobulin isotypes, IgG4 always appeared in the forefront as an immune tolerance-linked antibody isotype particularly induced by IL-10[31].

The mechanisms of NT development were investigated in two ways. First, it was compared with the allergic situation before OIT, and second, it was compared with tolerance induced after OIT. The main finding was a similar downregulation of many B cell genes in NT. A type 2 immune response (*IL4R* and

IL13RA1) is characteristic of allergic response as also observed in food allergy and a costimulatory receptor (*CD40*) of B cells were significantly lower in NT, proposing the inhibition of allergic responses[32]. Similarly, *TAP1* which plays a role in the antigen-presentation of B cells was lower in NT suggesting that the antigen-presentation capacity of specific B cells was downregulated[33]. The other downregulated genes, such as *BATF, HRH2, TLR2*, and *ADA* that are associated with allergic diseases were all downregulated in NT[34, 35, 36]. *ADA* is also associated with elevated IgE in atopic and food allergic patients[37, 38]. NT decreases several B cell cytokines or cytokine receptors such as, *IL7R* that controls homeostasis of allergen-specific memory CD4+T cells in the lung and airways[39], *IL12RB1*and *IL12RB2* that contribute to allergen-induced airway inflammation in asthma[40] *IL18R1* that is involved the pathophysiology of allergic diseases[41], and *IL23A* enhances Th2 polarization and regulates allergic airway inflammation[42]. As a part of specific B cell suppression in NT, chemotaxis genes including *PLAU* known as Treg suppressor, *CXCR3* that binds to chemokine CXCL-10 and enhances antigen-specific Th1 responses in healthy humans[43], *XCL1* and *XCL2* that play a crucial role in intestinal immune homeostasis[44], along with chemokine genes *CCL3L3, CCL4L2, CCRL2* that control airway inflammatory responses are all down regulated[45]. In addition, *GZMB* and *GZMH* that act as mediators of allergic inflammation in human asthma[46], *TGFB3* and its family gene *ACVR1B* that act in the initiation and effector phases of allergic disease, as well as in consequent tissue dysfunction[47], and *PIK3R3* gene that also signals in B cell co-stimulation were also down regulated[48].

Some of the genes that contribute to the B cell activation were upregulated, such as *NOTCH2* that suppresses food antigen-induced mucosal mast cell hyperplasia[49] and mediates the development and plasticity in marginal zone B cell[50, 51]. *FOXP1* that plays a critical role in human plasma cell differentiation was upregulated[52]. Two cytokine receptors *IL21R* and *IL2RG* via STAT3-dependent induction of transcription factors required for B cell expansion and plasma cell generation were increased in specific cells in NT[53]. The *TNFRSF1B*, a member of TNFRSF family, which acts as a co-signalling chemokine in BAFFR system for the B cell activation was increased in NT[54]. Toll-like receptors genes expression (*TLR1, TLR6, TLR9*) on B cells in NT, displayed the potential to induce secretion of cytokines, chemokines, and regulate immune homeostasis[55]. In addition, TLR9 is an important stimulus for Breg cell development[22]. CD22 was increased in NT, is an essential B cell suppressor molecule[56] which plays a role in allergen tolerance[57]. *PIK3CD* was also increased in NT, suggesting a role in the control of B cell development and function[58]. The significantly increased gene in specific B cells, *BCL6* suppresses IL-4 production in memory phenotype Th2 cells and suppresses Th2 immune responses in allergies[59]. *CARD11* mutations affect B cell development[60] and cause cellular defects in congenital lymphoproliferative patients and atopic dermatitis individuals[61, 62]. In CMA and house dust mite allergic patients, allergen-specific IgD was secreted after the course of OIT in serum samples[18, 63].

In the present study, we compared two types of immune tolerance to allergens, namely the natural and OIT-induced tolerance in specific B cells. One of the main differences between OIT-induced tolerance and

NT is that NT is a long-lasting tolerance, whereas OIT-induced tolerance has been recently established, and there is no guarantee that it will persist for long periods. It has been shown that viral infections can revert this kind of tolerance even in the course of OIT[64]. As a matter of fact, when we looked at the DEGs we can see that a significant number of genes show similar changes in expression in both OIT and NT samples compared to pre-OIT samples. When we focused on the genes that are different between the two types of immune tolerance, we observed *IL10RA*, *IGHG4, CD40, TAP1, IL23A, IL21, CD79A*, and *CD79B* are higher expressed in OIT-induced tolerance. *CD79A* and *CD79B* involved in membrane immunoglobulins to form the B-cell receptor complex[65]. This is an important observation, however the reasons why there is such a difference remain to be elucidated. In contrast, *STAT6, FOXP1 NOTCH2, TLR9, AHR, BCL6, CARD11, DOCK11, CD22, PIK3CA* are high in NT. Another supportive finding for more innate characteristic and suppressor function of specific B cells in NT and may play a role in long-term tolerance.

We also examined the secreted cytokines and surface receptors from αS_1-casein-specific B cells. These experiments were essential to characterize specific B cells in more detail. Since there were very low numbers of specific B cells, we had to immortalize and expand these cells. As a key finding of this group of experiments, IL-10 and LAP TGF-β were increased in NT and LAP TGF-β in OIT-tolerance compared to allergic individuals, demonstrating an important immune regulatory property gained by specific B cells. The majority of secreted proteins displayed characteristics of suppression of inflammation, such as TNFRSF9 marker of tumor-infiltrating regulatory T-cell in lung cancer[66], AXIN1 dysregulates Wnt pathway in adult eosinophilic esophagitis[67], uPA modulate airway eosinophilia[68], STAMBP inhibiting cytokine secretion through the NLRP3 and NLRP7 inflammasomes[69, 70], osteoprotegerin as suppressor of bone resorption and allergic asthma and rheumatoid arthritis[71], MMP-1 associating with bronchial hyperresponsiveness in asthma[72], Flt3L cytokine that prevents allergic asthma in mouse model[73], CASP-8 mediates IL-1 signalling in promoting Th2 responses in asthma[74], CSF-1 suppresses sensitization to aeroallergens and allergic lung inflammation in asthmatic mice[66], were increase in NT, suggesting the reduction of inflammation. These molecules are multifunctional, however, their real role in immune tolerance remain to be further analysed. Furthermore, similar increased protein expressions were observed in induced-tolerance after OIT and NT, such as TGF-β, TNFRSF9, AXIN1, uPA, STAMBP. Some of the proteins were increased in induced tolerance but not in NT. For example, CXCL10, CCL20 that activates Th2 cells and the expression of allergic airway disease[75]. VEGF-A plays a role in allergen-induced nasal inflammation[76]. IL-6, MCP-1[77] and MCP-4[78] that are associated with asthma susceptibility and severity and MMP-10 that contributes to airway remodelling in asthma[79]. ARTN that causes hypersensitivity in atopic dermatitis[80]. Overall, these data show that although there are similarities in NT and OIT-induced tolerance, there seem to be slightly different underlying mechanisms involved.

In conclusion, we demonstrated a detailed characterization of the transcriptome, secreted proteins, and secreted specific antibodies of allergen-specific B cells in cow's milk OIT-induced tolerance and NT individuals. We demonstrate that allergen-specific B cells show distinctive changes in induction of desensitization as well as induction of tolerance. The transcriptomic changes in specific B cells in OIT-

induced tolerance are one step further silencing of genes of B cell activation after desensitization. More complete and more numerous gene expression mechanisms of suppression are exhibited in OIT-induced tolerance when compared to desensitized individuals. A proinflammatory environment is observed in allergen-specific B cells in allergic individuals due to type 2 cytokine-related genes. This proinflammatory environment is altered in specific B cells that gain a suppressor capacity after OIT. There are similarities and differences in children that outgrow food allergy compared to OIT-induced tolerance. B cells appear to have one step further differentiation to pre-plasma stage with the expression of more innate immune receptors in NT. Breg cell-related genes are still active in OIT-induced tolerance with higher expression of *IGHG4, IL10* and *TGF-β* genes. Altogether, our data demonstrated that allergen-specific B cells are induced during OIT and natural tolerance and that they have some major gene expression changes that suggest an important role in induction, and maintenance of immune tolerance to food antigens.

Materials And Methods

Subjects

A total of twenty-four children with CMA who received oral OIT and children who naturally outgrew cow's milk allergy were recruited for this study. The subjects were assessed by a double-blind placebo-controlled food challenge (DBPCFC) as previously described[81, 82 83]. For the CMA group, PBMC were collected and biobanked at 0 months before the immunotherapy, during OIT 6-8 months, and 18-35 months ($n_{\text{before OIT, during OIT, after OIT}}=9$), and 4 additional patients after receiving 18-35 months immunotherapy timepoint ($n_{\text{after OIT}}=4$, $n_{\text{after OIT total}}=13$). Within a total of 13 patients from after OIT group, they were characterized as desensitized and tolerance ($n_{\text{after OIT (desensitized)}}=6$, $n_{\text{after OIT (tolerance)}}=7$). A group of children who had presented with previous allergic symptoms before and naturally outgrew cow's milk allergy and developed clinical tolerance was also included ($n_{\text{natural tolerance (NT)}}=9$). PBMC samples were biobanked at the Stanford University Sean Parker Asthma and Allergy Center, California, USA. Each participant with CMA older than 4 years was eligible for inclusion if they had proven sensitivity to milk documented by both a skin prick test greater than 3.5mm (wheal) and specific IgE greater than 2kU/L to cow's milk extract. Six weeks after the immunotherapy, participants withdrew the treatments and were challenged with cow's milk extract again. The treatment outcomes were defined based on allergic reaction results from cow's milk extract re-challenge at six weeks after the immunotherapy. Patients who showed no reaction up to 32.7 mg dose during the challenge, but began to show reactions at this dose were classified as desensitized. Patients who tolerated the 8,000-12,000 mg dose without any clinical reactions were identified as tolerance patients. Further characteristics of participants are listed in Table 1. All participant materials have been biobanked under the ethical permissions of local institutional ethical committees (Ethical permit number: IRB 8629, Sean N. Parker Center for Allergy & Asthma Research at Stanford University and Division of Pulmonary, Allergy, and Critical Care Medicine, Stanford University, CA, USA) and the study was conducted following the Helsinki Declaration. As a control, we included 9 healthy individuals between 27-40 years of age who had no allergic symptoms and consumed cow's milk ($n_{HC}=9$).

Labelling the allergen

The purified major allergen αS_1-casein from cow's milk was a kind gift from Dr. Els Van Huffen (NIZO food research, Ede, The Netherlands). αS_1-casein is a heat-stable and highly allergenic cow's milk allergen. This purified protein was confirmed with the ToxinSensor^{TM} gel clot endotoxin assay kit (GenScript, NJ, USA) as Lipopolysaccharide (LPS)-free. Afterward, αS_1-casein was labeled with a 20-fold molar excess of biotin reagent that resulted in 4-6 biotin groups per antigen molecule according to manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). The molar ratio of Sulfo-NHS-LC-Biotin to αS_1-casein was adjusted with titration experiment. Biotinylated αS_1-casein was titrated and used at a concentration of 0.625 µg for the labeling of αS_1-casein-specific B cells.

Food allergen-specific B cells isolation with flow cytometry

For the isolation of αS_1-casein specific B cells, biobanked peripheral blood mononuclear cells (PBMCs) were thawed and stained using zombie yellow viability dye (Biolegend, San Diego, CA, USA), washed in staining buffer (PBS, pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA), and resuspended in 100 µL staining buffer. Then, cells were incubated with antibodies against the surface markers CD19 (APC-eflour 780, clone: HIB19, ebioscience, San Diego, CA, USA), CD27 (AF700, clone: M-T271, Biolegend, San Diego, CA, USA), IgG (Dy405, goat-anti-human IgG, Sanquin, Amsterdam, Netherlands), IgA (AF488, goat-anti-human IgA, Jackson Immuno Research Europe Ltd, Cambridgeshire, UK) and IgM (PerCP/Cy5.5, clone: MHM-88, Biolegend, San Diego, CA, USA) at 4°C for 15 min. After a washing step, cells were incubated with 0.625ug/ml biotinylated αS_1-casein and subsequently incubated with the combination of streptavidin PE (BD bioscience, San Jose, CA, USA) and streptavidin Alexa fluor 635 (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C for 15 min. αS_1-casein-specific B cells were gated as ZombieYellow$^-$ CD19$^+$ αS_1-casein-PE$^+$alexa fluor 635$^+$ and purified using a FACS ARIA III cell sorter (BD bioscience, San Jose, CA, USA). The summary of the antibodies panel used in the experiment is shown in supplementary table 5. The enrichment sorting gate of αS_1-casein specific B cells were shown in supplementary Fig 2. Data were analysed with Flowjo, LLC Software (version 10, San Jose, CA, USA).

Immortalisation of food allergen-specific B cells

Both purified αS_1-casein-specific and non-specific B cells were transduced with a retroviral vector containing green fluorescence protein (GFP), *BCL6*, and *Bcl-xL* (a kind gift from AIMM Therapeutics, Amsterdam, the Netherlands) and cultured in IMDM medium (Thermo Fisher Scientific, Waltham, MA, USA) with CD40L-expressing L cells in the presence of IL-21 for 36 hours. These long-living B cells stably express functional BCR, display a memory phenotype, and produce antibodies and cytokines, thus offering many experimental advantages compared to Epstein-Barr virus (EBV) transformed B cells.[84] The combination of *BCL-6* and *BcL-xL* overexpression and the CD40L/IL-21 culture system was described previously[85 86].

Food allergen-specific antibody detection

5 µg/ml αS_1-casein was coated to a Nunc Maxisorb microtiter plate (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight and then blocked with blocking buffer (PBS pH 7.4, 5% BSA, 0.05% Tween 20). Supernatants from αS_1-casein-specific B cell samples were added and incubated for 2 hours at room temperature. Specific IgE was detected using a biotinylated anti-human IgE Abs (Kind gift of Dr. C. Heusser, Novartis, Basel, Switzerland), followed by streptavidin-peroxidase (Sigma-Aldrich, St. Louis, MO, USA). For the detection of specific IgG, goat anti-human IgG-peroxidase (Jackson Immuno Research Europe Ltd, Cambridgeshire, UK) was used. For specific IgG1 and IgG4 subclass detection, biotinylated anti-human IgG1 (clone: G17-1 RUO, BD bioscience, San Jose, CA, USA) or biotinylated anti-human IgG4 RJ4 Abs (Abingdon Health, York, UK) were used followed by incubation with streptavidin-peroxidase conjugated (Sigma-Aldrich, St. Louis, MO, USA). For specific IgG2 and IgG3 subclass detections, mouse anti-human IgG2 (clone: MH162-1, HP 6014, Sanquin, Amsterdam, the Netherlands) and mouse anti-human IgG3 (clone: MH163-1, HP 6095, Sanquin, Amsterdam, the Netherlands) were labeled with biotin (Sigma-Aldrich, St. Louis, MO, USA) and used as the primary detection antibodies. For specific IgA1 and IgA2 subclass detections, mouse anti-human IgA1 (clone: B3506B4, Abcam, Cambridge, UK) and mouse anti-human IgA2 (clone: B3506B4, Abcam, Cambridge, UK) were labelled with biotin (Sigma-Aldrich, St. Louis, MO, USA) and used as the primary detection antibodies. Goat anti-Human IgD Antibody HRP Conjugated (clone: A80-106P, Bethyl Laboratories, Montgomery, TX, USA) was used for the detection of specific IgD. ELISAs were developed using tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific, Waltham, MA, USA) and the reaction was stopped with 1M H_2SO_4 sulfuric acid at OD450 nm. Plates were read at 450 nm by a Mithras LB 940 spectrophotometer (Berthold Technologies, Bad Wildbad, Germany).

Library preparation and next generation of ultra-low input RNA-sequencing

Next generation RNA-sequencing library development includes cell lysis, reverse transcription into the first-strand cDNA, second-strand synthesis, and cDNA amplification. Sorted αS_1-casein-specific B cells of children with cow's milk allergy before OIT, after OIT, and children who spontaneously outgrew food allergy were lysed. Purified total RNA (100 ng) was ribosome depleted and then reverse-transcribed into double-stranded cDNA, with actinomycin added during first-strand synthesis. cDNA samples were fragmented, end-repaired, and polyadenylated before ligation using the SMART-seq® V4 ultra® low input RNA kit (Takara Bio, Mountain View, CA, USA). The cDNA was amplified with 11 cycles of PCR. To achieve the highest purity of cDNA, PCR clean-up was performed using Agencourt AMPure XP beads (Beckman Coulter GmbH, Krefeld, Germany). The quality and quantity of the cDNA were determined with a Qubit 1.0 Fluorometer (Life Technologies, South San Francisco, Calif) and the Bioanalyser 2100 (Agilent Technologies, Waldbronn, Germany). Then, the Illumina Nextera XT kit (Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA) was used to make cDNA libraries suitable for Illumina sequencing. The libraries were normalized to 10 nM in Tris-Cl 10 mM, pH 8.5 with 0.1% Tween 20. After that, sequencing was performed on the Nova seq (Illumina Way, San Diego, CA, USA). The sequencing results were quantified in read-based terminologies, such as RPKM/FPKM (read/fragment per kilobase

per million mapped reads) and normalized counts at the Functional Genomic Center Zurich, University of Zurich, Switzerland.

RNA-sequencing data analysis

The quality control on raw sequence data was analysed with FastQC (version 0.10.0; Babraham Institute, Cambridge, United Kingdom) and mapped to the Homo sapiens genome (GRCh38 build) using RSEM (version 1.2.12) implementation of the Bowtie software (version 1.0.0) alignment program with the Ensembl annotation (version 75). Gene and isoform level abundances were quantified as reads per kilobase per million mapped read values. Clustering analyses were performed with the “ward.D2” clustering algorithm implemented in the “hclust” function of R statistics package. Heat map plots were performed with the function “heatmap.2” implemented in the gplots R package. Differential expression analysis between the 2 groups was performed with the edgeR Bioconductor package. Genes present in less than 75% of samples in both conditions were excluded. Q-values were calculated by using the Benjamini-Hochberg method, and genes with a q-value of less than 0.015 and an absolute log2 (fold change) value of greater than 1 were kept for further analysis. Gene Ontology term enrichment analysis was performed with the GOseq Bioconductor package using the Wallenius approximation. Pathway analysis was done with MetaCore version 6.3 (Thomson Reuters) Enricher platform with the Panther database. Significant pathways were defined as pathways with an adjusted p-value of less than .01. Gene lists were created as described in the text or based on gene lists from NIAID ImmPort Resources (http://www.immport.org/immport-open/public/reference/genelists). Genes with p-value <= 0.01 and log2 fold change >= 0.5 were used in this study. Gene network enrichment was generated using MetaCore version 6.3 (Thomson Reuters), and identification of shared genes was analysed by Venn diagram tool (FGCZ VennTool, functional genomics center Zurich, Switzerland). Data was uploaded to GEO database as PRJEB48819.

Proximity extension assay (PEA)

The PEA with Olink inflammatory panel (Olink Biosciences, Uppsala, Sweden) used in this study includes 92 proteins and for each sample, 1 µL of cultured B cell supernatants was used. The PEA method is achieved by the binding of PEA probes on paired antibodies from the samples. The first step is the immune reaction in which 92 Abs pairs, labeled with DNA oligonucleotides, bind to their respective protein in the samples during 16-22 hours incubation. Next, extension and pre-amplification were performed in which oligonucleotides were extended using a DNA polymerase. This newly created piece of DNA barcode is amplified by PCR for 2 hours. Then, detection in which the amount of each DNA barcode is quantified by microfluidic qPCR in 4 and a half hours. Finally, the detection of proteins of interest was examined through quantitative real-time PCR. The PCR results were evaluated as normalized protein expression (NPX) values, which are arbitrary units on a $\log_2$ ratio. NPX is calculated from Ct values and data pre-processing is performed to minimize both intra- and inter-assay variation. A high NPX value corresponds to a high protein concentration and expresses relative quantification between samples but represents no absolute quantification. Assay characteristics including quality control, detection limits, and

measurements of assay performance and validation were done at the Olink reference center, the Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland.

Statistical analysis

Data were expressed as a mean ± standard error of the mean (SEM). Statistical analysis was conducted using GraphPad Prism 7.0 Software (GraphPad Software, La Jolla, CA, USA). Gene expression between multiple groups was analysed by using the Wilcoxon signed-ranked test, and comparison between groups was done with the Mann-Whitney test. A p-value of less than 0.05 was considered statistically significant.

Declarations

Author contributions

P.S. carried out the experiments of the study, data analysis, and manuscript preparation. W.V. and M.A. provided critical inputs in designing the study and interpretation of results. G.T. conducted a statistical analysis approach to RNA-seq data. P.S., W.V., G.T., O.F.W., K.J., M.S., S.D.B., C.A.A., K.N., M.A. contributed to the data analysis and interpretation of the results. D.M., A.G., T.B., S.R.S. assisted in performing the experiments. E.B. analysed the proximity extension data. H.S. provided knowledge regarding the immortalisation technique of allergen-specific B cells. I.C. recruited the participants and biobanked the PBMCs specimens. M.A. supervised the project. All authors contributed to the revision of the manuscript.

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Competing Interests

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Additional information

Supplementary information is available for this paper.

Abbreviations

Abs	Antibodies
CD	Cluster of differentiation
CD40L	CD40 ligand
CMA	Cow's milk allergy
cDNA	Complementary DNA
Bcl-6	B-cell lymphoma 6
Bcl-xl	B-cell lymphoma-extra large
BCR	B cell receptor
Breg	B Regulatory
DEGs	Differentially expressed genes
OIT	Oral allergen-specific immunotherapy
GFP	Green fluorescence protein
HC	Healthy controls
Igs	Immunoglobulins
IL	Interleukin
LPS	Lipopolysaccharide

NT	Natural tolerance
PBMCs	Peripheral blood mononuclear cells
PEA	Proximity extension assay
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
sIgE	Specific immunoglobulin E
sIgG	Specific immunoglobulin G
sIgG1	Specific immunoglobulin G1
sIgG4	Specific immunoglobulin G4
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor

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Table 1

Table 1 is available in the Supplementary Files section.

Figures

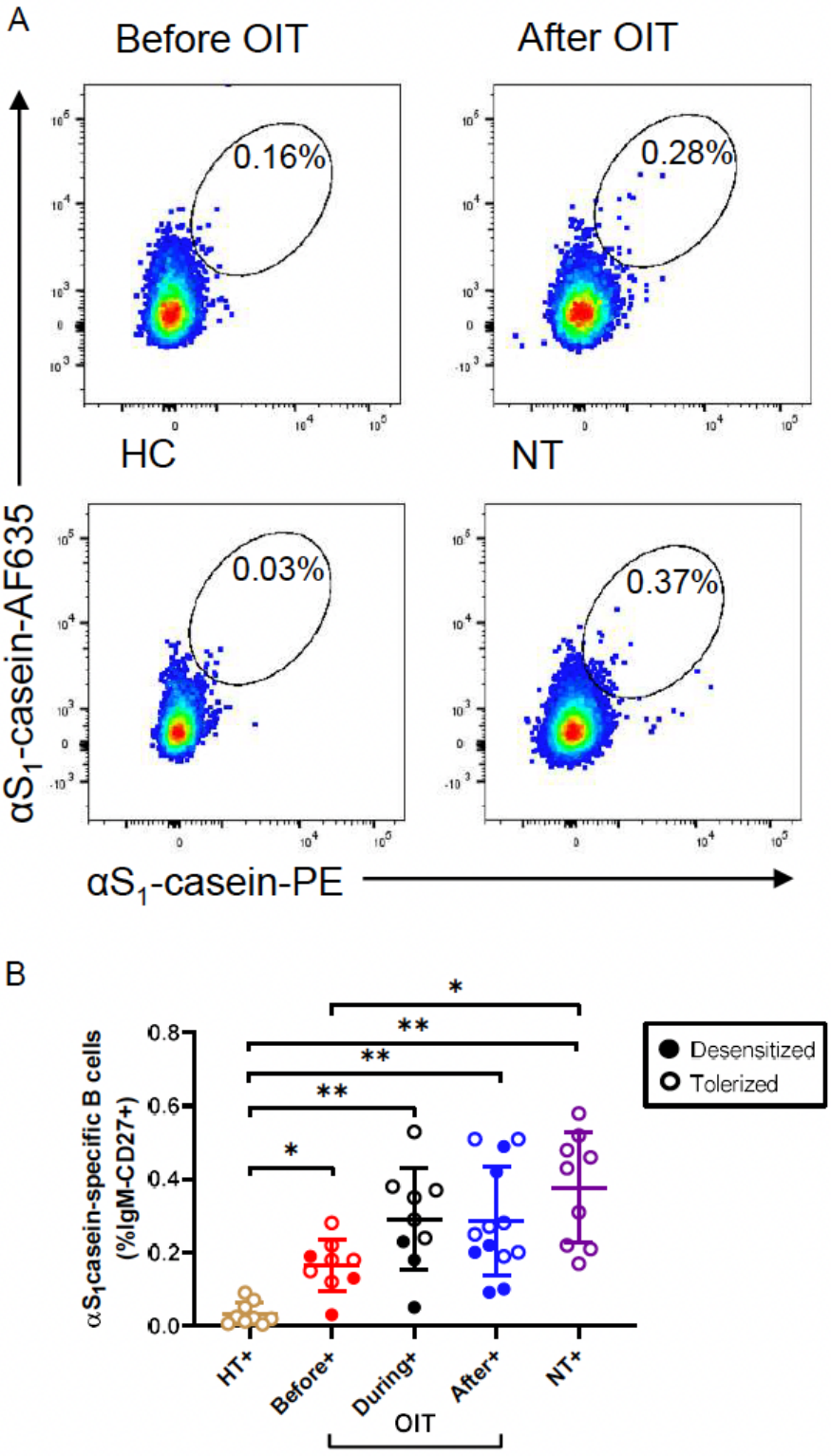

Figure 1

The frequency of αS$_1$-casein-specific B cells is increased after OIT in allergic patients and natural tolerance compared to healthy individuals.

Flow cytometry plots in (A) are representative data coming from 4 samples; before OIT, after OIT, HC, and NT, respectively. (B) Frequencies of αS$_1$-casein-specific B cells was analysed by one-way ANOVA, * p<0.05,

** p<0.01; *** p<0.001, **** p<0.0001; Values are means ± SEM, bold dots represent “desensitized” and empty dots represent “tolerance” (n_{HC}=9, $n_{before\ OIT}$=9, $n_{during\ OIT}$=9, $n_{after\ OIT}$=13, $n_{natural\ tolerance\ (NT)}$=9)

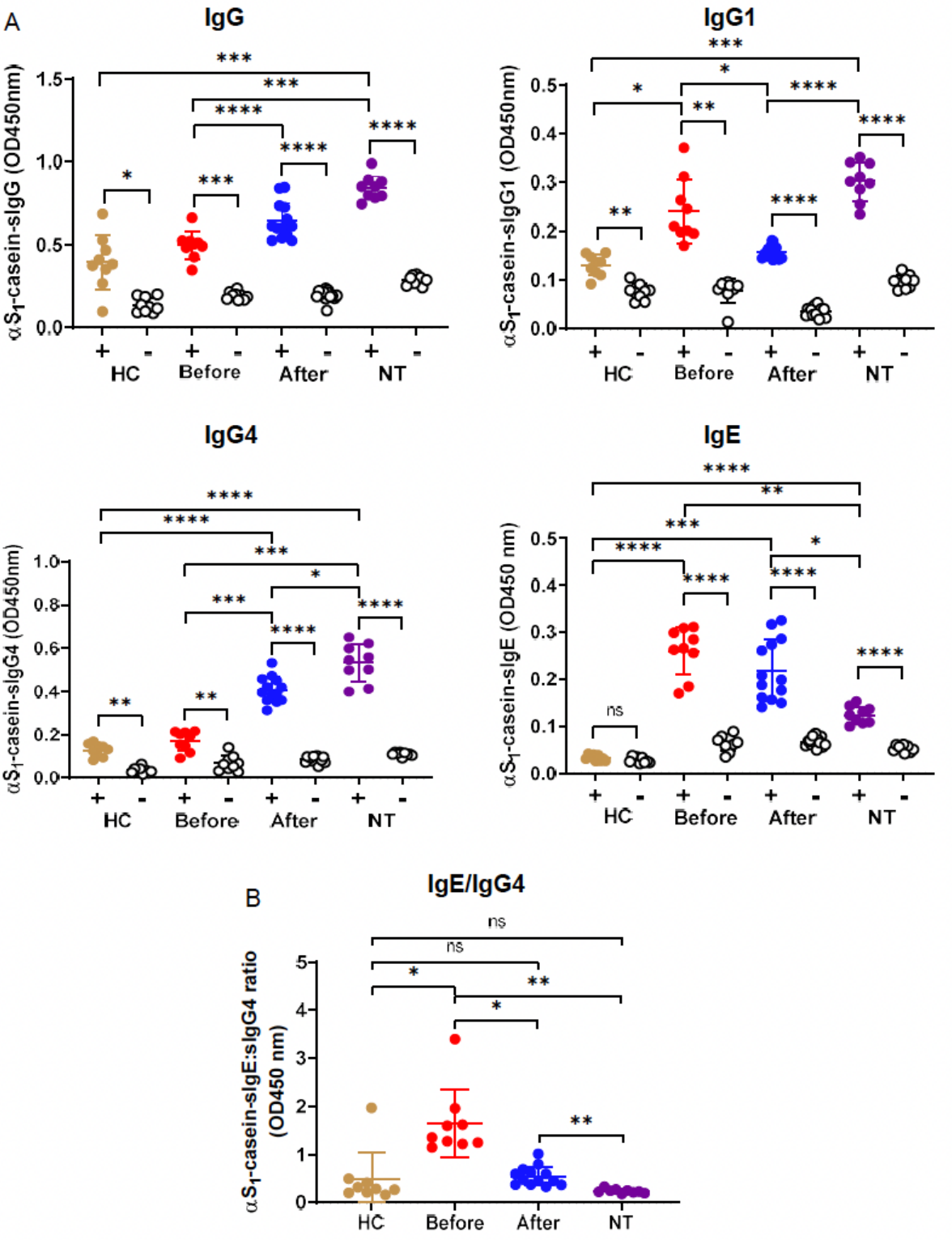

Figure 2

Specificity of αS_1-casein-specific immunoglobulin detection in allergic patients undergoing OIT versus natural tolerance and healthy controls.

(A) Detection of αS_1-casein-specific immunoglobulins in supernatants of αS_1-casein-specific and non-specific immortalized B cells (500,000 cells/ well) at day 3; sIgG, sIgG1, sIgG4, sIgE. (B) αS_1-casein-specific sIgE/sIgG4 ratio analysed by one-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; Values are means ± SEM, ($n_{HC}=9$, $n_{\text{before OIT}}=9$, $n_{\text{after OIT}}=13$, $n_{\text{natural tolerance (NT)}}=9$).

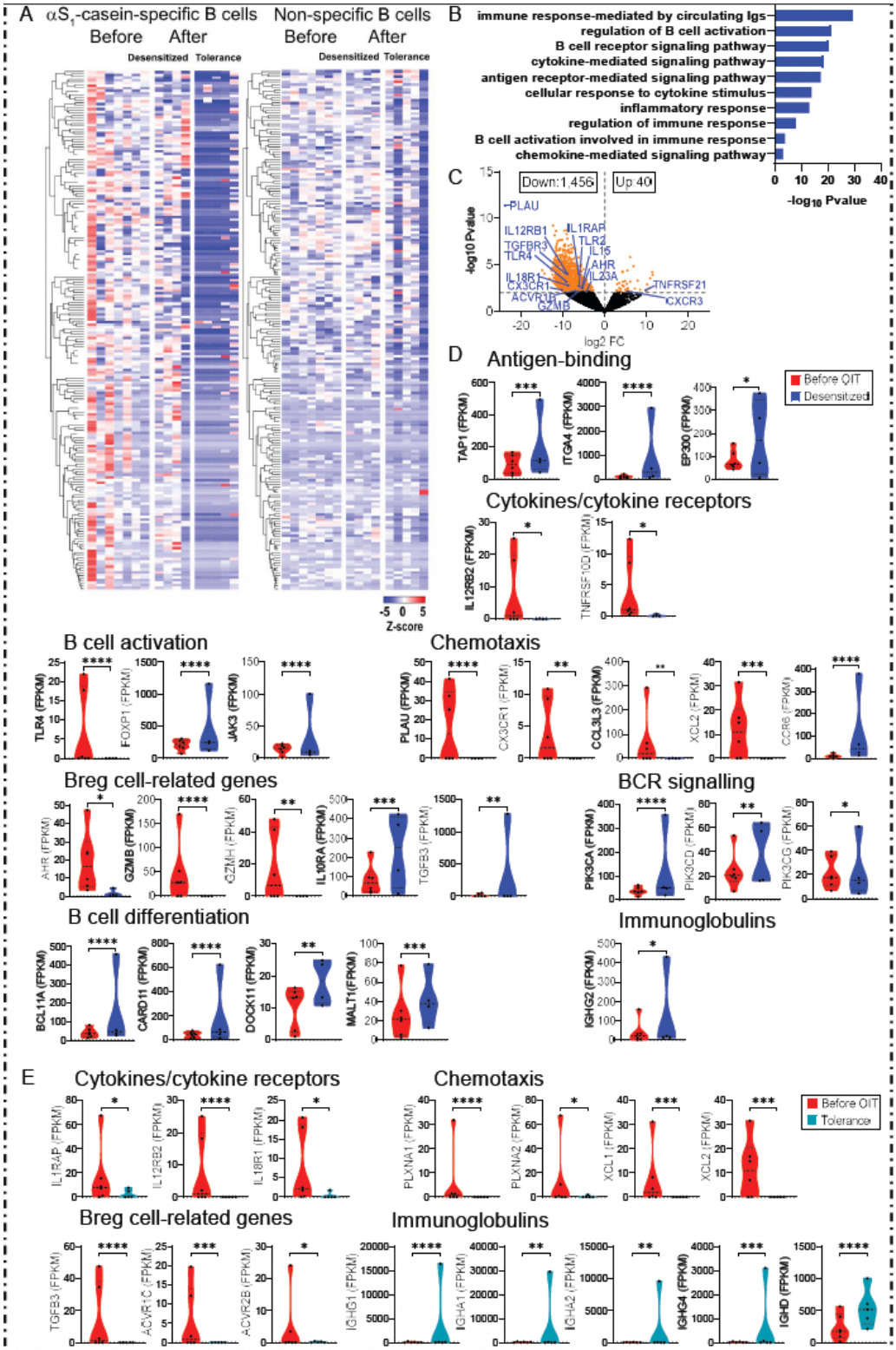

Figure 3

DGEs signatures in αS_1-casein-specific and non-specific B cells before and after OIT (desensitized & tolerance).

(A) Heatmap of the top 200 most DGEs in αS_1-casein-specific and non-specific B cells from before and after OIT, $n_{\text{before OIT}}=6$, $n_{\text{after OIT (desensitized)}}=4$, $n_{\text{after OIT (tolerance)}}=5$ (B) Top B cell-related pathways analysis

(C) Volcano plot shows differentially expressed genes with top 20 significant genes (p-value <0.05). Violin plots of differentially expressed genes in 8 different gene groups; antigen-binding, cytokines/ cytokine receptors, B cell activation, chemotaxis, Breg cell-related genes, BCR signalling, B cell differentiation, and immunoglobulins; (D) before OIT vs desensitized and (E) before OIT vs tolerance, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

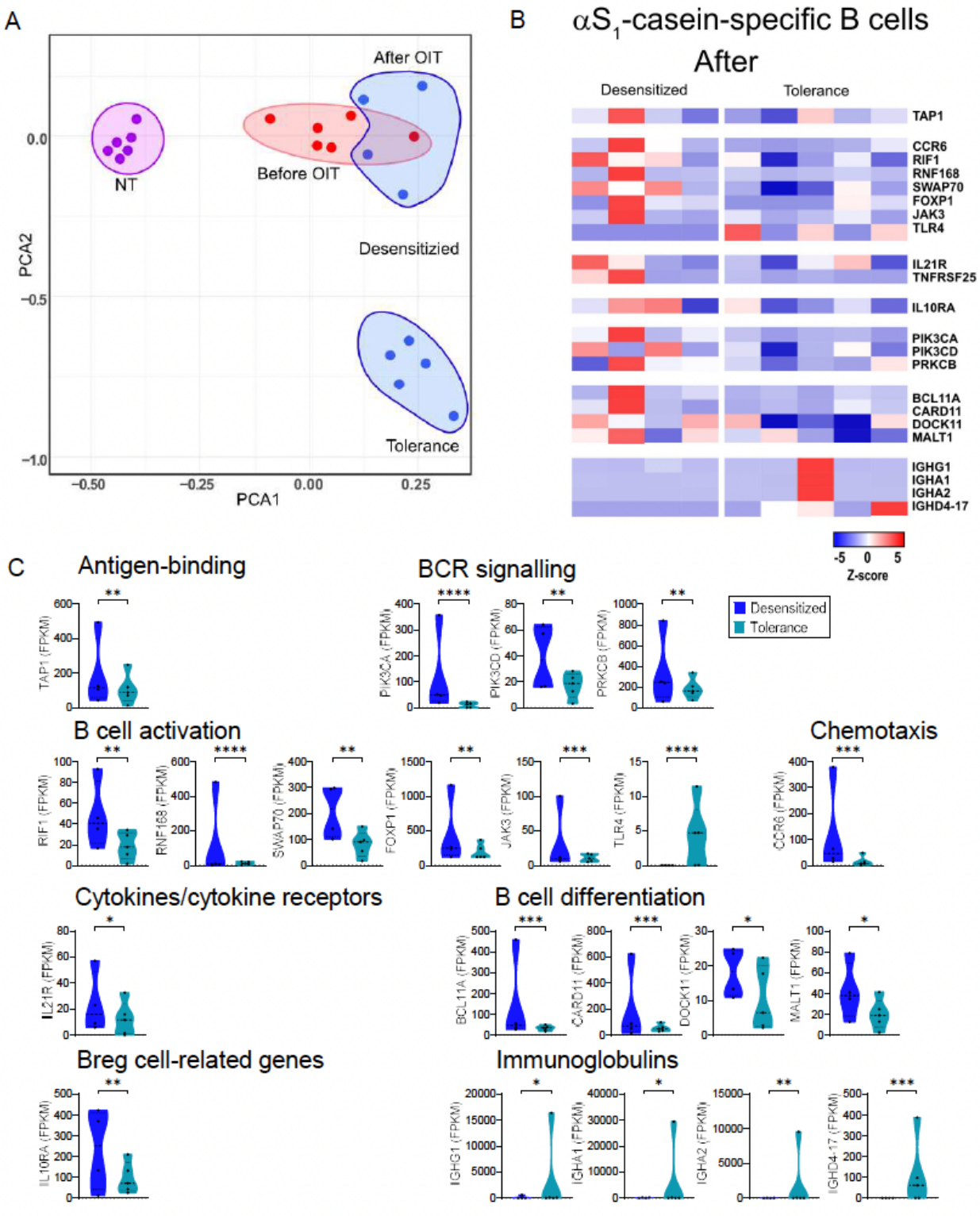

Figure 4

DGEs signatures in αS_1-casein-specific B cells from after OIT (desensitized vs tolerance).

(A) PCA plot of 200 DGEs separating allergic groups: before OIT vs after OIT (desensitized and tolerance) vs NT groups. (B) Heatmap of most significant DEGs of αS_1-casein-specific B cells after OIT; desensitized VS tolerance, $n_{before\ OIT}$=6 and n_{NT}= 6 (C) Violin plots of differentially expressed genes in 8 different gene groups; antigen-binding, BCR signalling, B cell activation, chemotaxis, cytokines/ cytokine receptors, B cell differentiation, Breg cell-related genes, and immunoglobulins, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

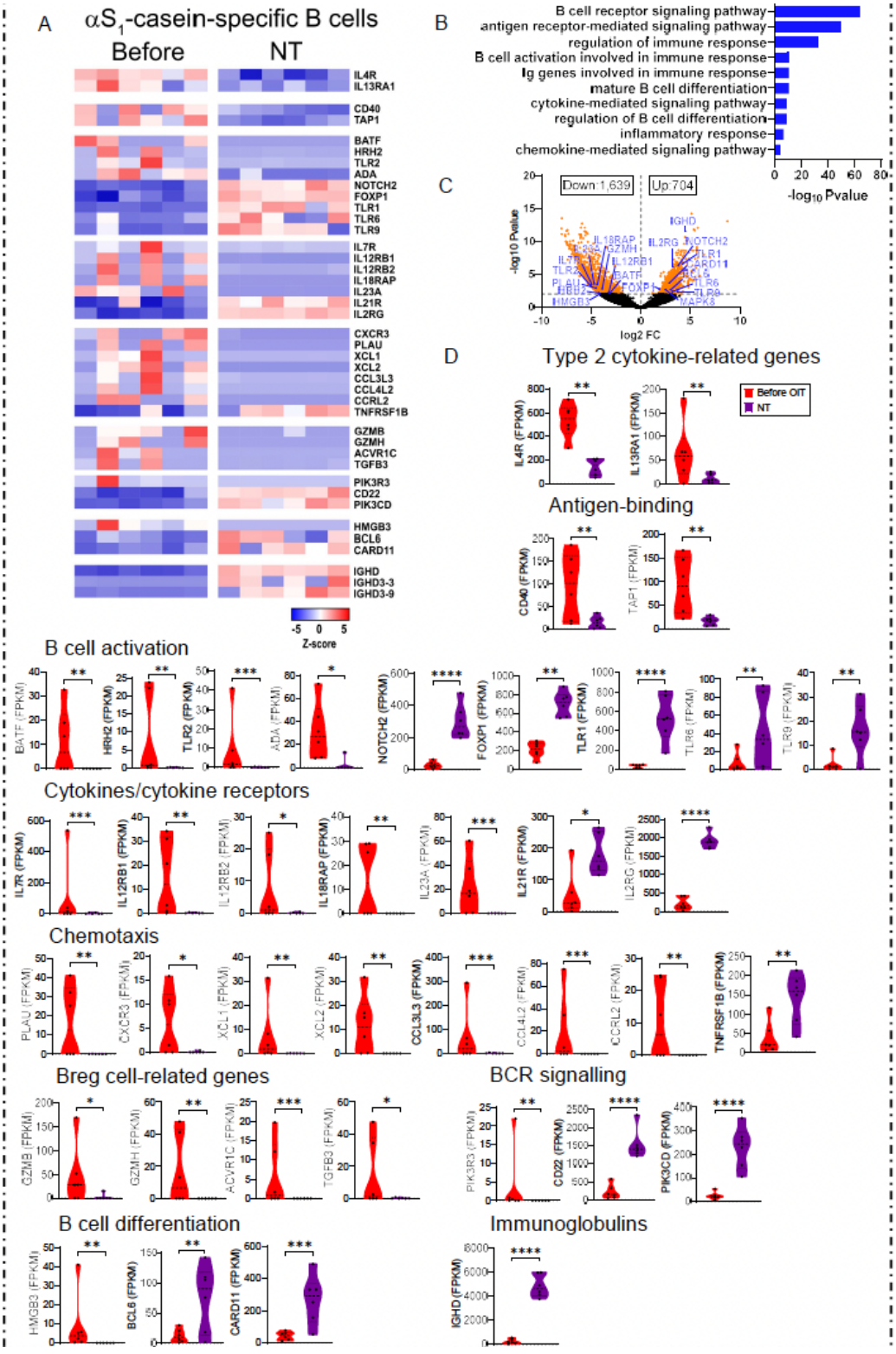

Figure 5

DGEs signatures in αS_1-casein-specific B cells from before OIT vs NT.

(A) Heatmap showing top significant DEGs of αS_1-casein-specific B cells from before OIT and NT, $n_{before\ OIT}$=6 and n_{NT}= 6. (B) Top B cell-related pathways analysis (C) Volcano plot shows differentially expressed genes with top 20 significant genes (p-value <0.05). (D) Violin plots of differentially expressed

genes in 9 different gene groups; Type 2 cytokine-related genes, antigen-binding, B cell activation, cytokines/ cytokine receptors, chemotaxis, Breg cell-related genes, BCR signalling, B cell differentiation, and immunoglobulins, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

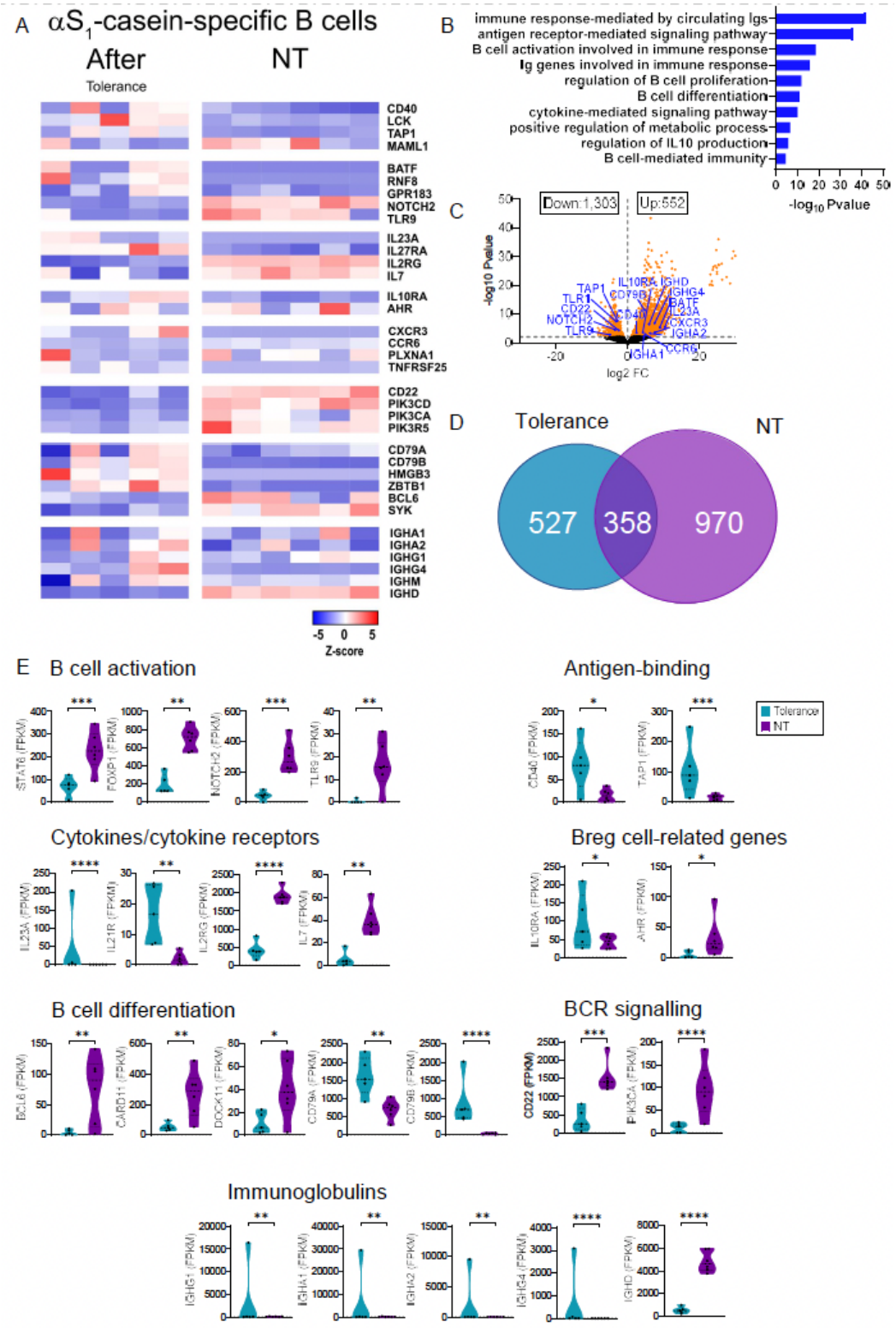

Figure 6

DGEs signatures in αS_1-casein-specific B cells from after OIT (tolerance) vs NT.

(A) Heatmap of the top significant DEGs of αS1-casein-specific B cells from after OIT (tolerance) vs NT, n_{after} OIT (tolerance)=5, n_{NT} OIT=6, (B) Top B cell-related pathways analysis (C) Volcano plot shows differentially expressed genes with top 20 significant genes (p-value <0.05). (D) Venn-diagram of differentially expressed genes from tolerance vs NT groups (p-value <0.05). Violin plots of differentially expressed genes in 7 different gene groups; B cell activation, antigen-binding, cytokines/ cytokine receptors, Breg cell-related genes, B cell differentiation, BCR signalling, and immunoglobulins; (E) tolerance vs NT, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

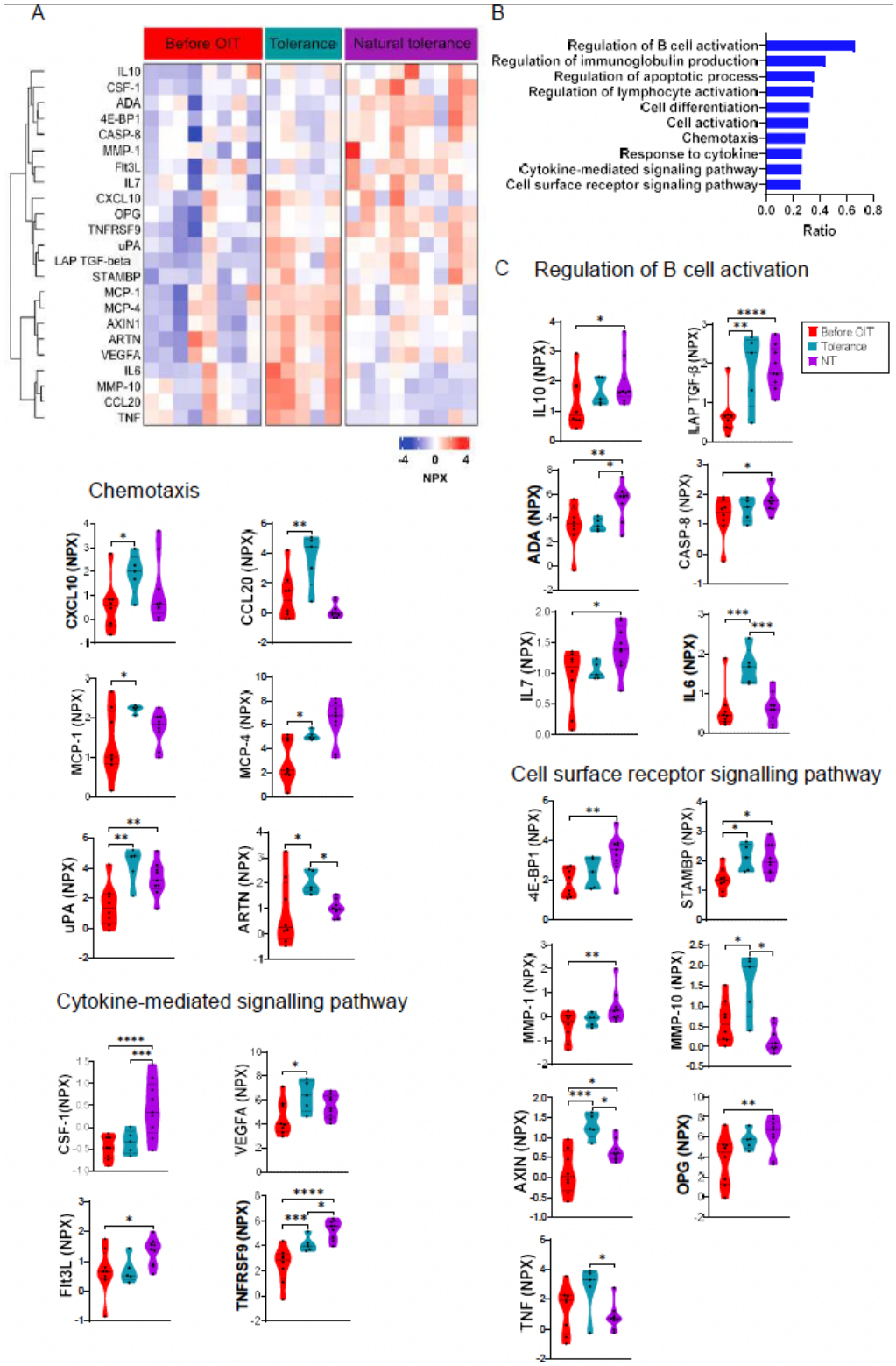

Figure 7

Protein expressions in αS_1-casein-specific B cell cultures from before OIT, after OIT (desensitized & tolerance), and NT using proximity extension assay (OLINK).

(A) Heatmap depicts significant differentially secreted proteins from αS_1-casein-specific B cell supernatants before OIT vs after OIT vs NT. (B) Ratio of 23 significant proteins, for different GO Biological

processes, to all measured in the Olink inflammation panel. (C) Violin plots of differentially secreted proteins were showed: IL10, TGF-β, TNFRSF9, AXIN, uPA, STAMBP, OPG, MMP-1, IL7, FlT3L, CASP-8, 4E-BP1, ADA, CXCL10, CCL20, VEGF-A, MCP-1, MCP-4, TNF, MMP-10, ARTN, CSF-1, and IL6, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AMAlphaS1caseinsupplementary.docx
- Supplementaryfigures.pdf
- Table1.docx