

# Tertiary lymphoid structures related B-cell IgE isotype switching and secondary lymphoid organs linked IgE production in mouse allergy model

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## Research article

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# Abstract

**Background:** Numerous data obtained by different research laboratories around the world indicate that specific IgE production is triggered independently of specific IgG or IgA production and so did not linked to fully matured germinal centers of secondary lymphoid organs. The aim of this study is to clarify whether specific IgE production is triggered by low antigen doses administrated in tertiary lymphoid structure enriched tissues.

**Methods:** OVA in different doses (100 ng or 10 µg) was administrated three times a week for 4–5 weeks intraperitoneally and subcutaneously to female BALB/c mice in the withers region enriched in fat-associated lymphoid clusters and in foot pad region not containing them.

**Results:** OVA-specific IgE was predominantly induced by low but not by high antigen doses and only after immunization in withers. IgE isotype switching was triggered exclusively in withers adipose tissue but not in regional lymph nodes though mature IgE expressing cells were observed both in tissue and lymph nodes. Anti-proliferative genotoxic stress inducing drugs shifted the balance from IgG1 towards IgE production.

**Conclusion.** Tertiary lymphoid structures possess unique environment where B-cell antibody isotype switching to IgE predominantly occurs. These phenomena are explained by hampered proliferation of B-cells in these structures.

## Background

In order to develop new methods of allergen-specific immunotherapy dedicated to stimulate allergen-induced B-cell switching to IgG4 but not IgE isotype, it is clearly necessary to understand specific mechanisms triggering selective B-cell immunoglobulin class switching to these isotypes. For development of the general strategy to eliminate IgE-producing B-cells and their precursors, it is important to know the exact site where B-cell IgE isotype switching occurs. We have previously shown that in young (1–8 years old) patients with allergy to house dust mite specific IgE production is not associated with specific IgG, IgG4 or IgA1 production [1] and may occur outside the germinal centers of secondary lymphoid organs (SLOs), for example, in the tertiary lymphoid structures (TLSs) or so-called tissue-associated lymphoid clusters. Because of the close proximity to epithelial or endothelial barrier tissues immune processes in TLSs may be somewhat different from those in SLOs. For example, type 2 innate lymphoid cells (ILC2) activated by tissue damage-induced cytokines IL-25, IL-33 and TSLP [2], instead of T helper-2 cells, may act as the main producers of pro-allergic cytokines like IL-5, 13 [2] and, to lesser extent IL-4 [3], in TLSs during the early phases of immune response.

Despite a large number of successively developed mouse allergy models, most of them required high allergen doses [4–9] sometimes with adjuvants like Alum [4–7] or CFA [8], and led not only to high IgE levels but also to high IgG1 [4–7] and sometimes IgG2a [9] production. So, in these models, humoral immune response differs from that in allergic patients. Among allergic individuals some of them have

significant IgG<sub>4</sub> production [1, 10, 11] which is not, however, associated with specific IgE levels [1, 10, 11]. Elevated IgG4 levels detected in atopic patients can represent organism's secondary reaction in response to multiple numbers of antigen entrance. The presence of already existing specific IgE makes it possible to induce IgG4 production due to immune recognition of IgE-antigen complexes. [12–14]. Indeed, recent data reviewed in [15, 16] shows that high antigen doses induce robust germinal center response in SLOs which could in certain circumstances block development of IgE producing B-cells. It is not surprising that B-cell IgE class switching is usually detected in the SLO-like lymph nodes and specifically within germinal centers but this process is transient, since it occurs mainly in early stages of its development and not in mature structures [17, 18]. Moreover, numerous studies [19–21] provide the evidence that in allergic patients B-cell IgE class switching usually occurs in TLSs such as nasal polyps or inducible bronchial-associated lymphoid clusters.

The aim of this work was to clarify the hypothesis that B-cell IgE class switching in response to low, but not high, administered antigen doses occurs mostly in the specific milieu of TLSs but not in SLOs. In clinical practice allergens usually pass through respiratory barrier tissues, and inducible bronchial-associated lymphoid tissue or nasal polyps represent TLSs [20, 22]. In humans these clusters are induced during early infancy, especially among the children with genetic defects in lung epithelial barriers, due to their higher susceptibility to viral and bacterial infection, or to exposure to certain substance such as diesel-derived particles [23]. These structures can be generated in infant mice by administration of specific substances such as LPS [24]. To avoid the difficulties of small antigen doses delivery to the mouse lungs as well as to induce respiratory tract linked TLSs formation in adult animals, we chose to use principally novel model in which antigen was administered in subcutaneous fat in the withers. It is well known that this area in mice contains significant volume of white adipose tissue and many so called fat-associated lymphoid clusters (FALCs) [25] which resemble those associated with respiratory epithelium. Importantly, tissue damage-induced cytokines, such as IL-33 and TSLP, could be produced not only in barrier epithelium cells [2] but also in adipose tissue, particularly, in adipocytes and adipose-linked endothelium. Of note, at first ILC2 were discovered in association with these FALCs [28], though later ILC2 were identified in SLOs as well [2]. In adipose tissues, ILC2 not only promote allergic type 2 inflammation but also function as metabolic and homeostatic regulators [29].

We demonstrate here that direct IgE class switch in response to subcutaneously administered allergen occurs in local TLSs, and adipose tissue provide unique environment for B cell activation.

## Results

### **Low dose antigen administration in TLS-enriched, but not TLS-depleted, zone induces specific IgE production.**

To reconstitute typical clinical situation in which allergens enter over a long time period in the organism, mice were repeatedly immunized with low (100 ng) and high (10 µg) antigen doses in the regions which are relatively enriched in TLSs (withers) or lack them (foot pad). Intraperitoneal administration was

carried out for comparison. For each experiment female 6–8 weeks old (18–25 g weight) naïve BALB/c mice were used. Mice were kept in SPF conditions. The results (Fig. 1A-F) show that specific IgE production was induced only after chronic antigen administration in withers region and mostly in low dose group. Specific IgE production was still significant after 7th immunization, and at this time point was comparable both in high and low dose groups. However, after 14th immunization it was markedly enhanced in low dose group and only slightly in high dose group. In contrast to this observation specific IgG1 production (Fig. 1G-L) was significant almost in all immunized mice groups. It is not surprising that high antigen doses induce more pronounced IgG1 production. This production was comparable between high dose groups, which probably indicate the same efficacy of antigen delivery to SLOs from all these regions. Nevertheless, chronically administered antigen doses induced significant though lower IgG1 production as well, and between low doses groups, it was more pronounced upon antigen administration in withers and less pronounced in i.p. and f.p. administered groups, according to titer values. Specific IgG2a production was minimal in all groups, and increased significant after prolonged (14th ) immunization with high antigen doses in withers region (Fig. 2) and was triggered only after prolonged, but not after short-time (7th ) immunizations.

### **The degree of mice sensitization by low antigen doses is associated mostly with specific IgE production.**

It is well appreciated that in humans type I hypersensitivity reactions mediated by mast cell and basophil mediators is linked exclusively with allergen-specific IgE antibodies. However, in mice IgG1- and even IgG2a- mediated type I hypersensitivity becomes possible because of basophils degranulation in response to IgG immune complexes [30]. In addition, PAF-mediated macrophage-dependent anaphylactic response which closely resembles histamine and leukotriene mediated reactions becomes possible as well [31]. As mentioned above, in most currently used allergic models, IgE production is triggered together with high levels of specific IgG1 production [4–9] which makes clinical situation in these models slightly different from that in human individuals. We presume that in our low-dose allergic model situation may be substantially different. To clarify this, we first measured systematic (Fig. 3A) and local (Fig. 3B) anaphylaxis intensity in mice after long-time administration of high and low antigen doses. Indeed, mice in low dose groups had more pronounced symptoms of local and systemic anaphylaxis. In the second step we estimated whether systematic anaphylaxis intensity ( $-dT$ , which could be easily measured) was associated with specific IgE or IgG1 production. Results (Figs. 3C and 3D) show that the degree of sensitization measurement by systematic anaphylaxis intensity is linked with both IgE and IgG1 production, but mainly associated with elevated IgE levels. During the course of allergic sensitization process not only specific but also total IgE levels rise significantly. But the impact of such IgE antibodies with undefined specificity on antigen-induced mast cell activation may be negative especially in the case when specific IgE production is low [32]. Indeed, systematic anaphylaxis intensity did not correlate with total IgE levels (Fig. 3E) but there was correlation with ratio between specific and total IgE levels (Fig. 3F). It is also need to be mentioned that despite the common mechanisms of induction, specific IgE are produced independently of IgG1 (Fig. 3G) and, surprisingly, the same is observed in case of total and specific IgE (Fig. 3H).

## **B-cell activation by low antigen doses occurs exclusively in tissue in the site of antigen administration.**

To confirm our hypothesis about predominant local (in the site of antigen administration) B-cell activation, we estimated expression of genes corresponding to B-cells in general (*cd19*), their activated state during germinal center formation (*Bcl6*) [33] or extrafollicular foci formation (*Ebi2*) [33], as well as to the class switch DNA recombination in general (*Aicda*) [34] or to the IgE and IgG1 switching (*germline ε* and *germline γ1* transcripts respectively) [34]. As shown in Fig. 4A B-cell immunoglobulin class switching induced by low antigen doses occurs exclusively in withers tissue. In low dose mouse groups, expression of *Aicda*, *germline ε* and *germline γ1* were triggered exclusively in withers tissue but not in regional lymph nodes. It is interesting that even high antigen doses did not trigger germline transcripts expression in lymph nodes though these doses trigger lymph nodes' *Aicda* expression. It should be mentioned that high but not low antigen doses induce relative B-cell accumulation in withers tissue. In contrast, low antigen doses induce B-cell depletion from withers tissue as evident from relative drop of *cd19* expression. Both doses of antigen induced comparable levels of extrafollicular B-cell activation marker (*Ebi2*) but low doses induce more pronounced germinal center formation in withers characterized by *Bcl6* upregulation. However, it is not surprising that high antigen doses induce significant accumulation of both types of activated B-cells in lymph nodes. It should be noted, that predominant expression of transcripts corresponding to B-cell activation in low dose group was observed not only when expression was normalized to *Gapdh* but also when it was normalized to *Cd19* (*data not shown*). B-cell IgE class switching depends on IL-4 and less on IL-13 which could be produced by either T helper 2 or ILC2 cells. Both cell types express transcription factor GATA3 [3, 35], but among all immune cells only ILC2 express NMUR1 [36]. We have measured expression of these genes in withers and lymph nodes. Low antigen doses induced substantial increase in *Gata3* and *Nmur1* genes expression in withers tissue. Only high antigen doses triggered expression of *Gata3* in lymph nodes (Fig. 4B). Long time antigen administration via needle induced damage of adipose tissue and, therefore, stimulated the expression of tissue cytokines. Expression of *Il25* (only in low dose group) and *Il33* (both groups) but not *TSLP* was induced (Fig. 4C). It should be noted that due to normalization to respective control samples taken from the same organ the intensity of expression between W and LN could not be directly compared. The comparison is performed only between the samples from the same tissue.

## **Specific IgE production predominantly occurs in regional lymph nodes after migration of activated IgE-switched B-cells from TLSs.**

In order to develop new allergen-specific immunotherapy methods based on elimination of IgE-producing B-cells and their precursors, one should entirely know not only the site where IgE antibody isotype switching occurs but also the site of IgE antibody production. Because B-cells from TLSs can recirculate between these structures and SLOs [37, 38] one could not exclude the possibility that the final differentiation of IgE<sup>+</sup> B-cells into antibody producing cells takes place not only in TLSs but also in SLOs. Indeed, data from Fig. 6 indicate that expression of *postswitch ε* and *postswitch γ1* transcripts was induced in regional lymph nodes. It is not surprising that in case of regional lymph nodes *postswitch ε* and *postswitch γ1* transcripts were mainly induced by low and high antigen doses, respectively,

regardless of reference gene, i.e. *Gapdh* or *Cd19*, the expression of genes of interest was normalized to (Fig. 5B and 5D). However, in adipose tissue the effects of low vs high doses on genes expression were different. When normalized to *Gapdh*, the predominant expression of *postswitch ε*, rather than *postswitch γ1*, in high, rather than low, dose groups was evident (Fig. 5A and 5C). When normalized to *Cd19*, the expression of *postswitch ε* transcripts was comparable in both groups (Fig. 5B and 5D). Despite inefficient IgE class switch in adipose tissue observed in high dose group, and considering absolute number of B cells rather than percentage of total B cell population, the accumulation of IgE expressing B cells in tissue was more prominent in high vs. low dose group.. Together with the data that indicates B-cell depletion from adipose tissue in low but not high dose group (Fig. 4A), our results suggest that activated B-cells migrate from TLSs to SLOs.

### **Dampening of cell proliferation by anti-proliferative drugs enhances specific IgE but not IgG1 production.**

One of the most interesting observations from data described above is that expression of *cd19* marker in adipose tissue is downregulated in low dose group compared to control and high dose group, despite the fact that B-cell activation in withers occurs mostly in low dose group. In contrast, relative CD19 accumulation in withers of high dose group was accompanied by relatively weak intensity of B-cell activation and IgE class switching. Because of the harmless nature of OVA diluted in saline the administration of low antigen dose is unlikely to induce necrotic or apoptotic death of B-cells. It is known that activated B-cells tend to migrate between TLSs and SLOs and the data shown in Fig. 4 support this point of view [39]. Upon antigen-based activation, B-cells tend to proliferate as well but the number of B-cells' niches in TLSs must be more limited than in SLOs. So, it is reasonable to suppose that restriction of proliferation caused by limited number of niches (or limited proliferation supporting factors) in TLSs in contrast to SLOs shifts the balance in former from IgG1 to IgE production by yet unknown molecular mechanism. To verify this, we used two commercially available anti-proliferative drugs doxorubicin and etoposide. The water-soluble doxorubicin can be rapidly distributed in internal milieu. Etoposid is more hydrophobic and its specific action would be probably more restricted to adipose tissue. Drugs were administrated with antigen for long time period. Data from Fig. 6A-D indicate that both drugs induced significant ( $p < 0.05$ ) increase in OVA specific IgE production after prolonged (14th immunizations) treatment, though only high (20  $\mu\text{g}/\text{injection}$ ) dose had effect. After short time treatment (7th immunizations) only etoposide's effect was significant. In contrast, specific IgG1 production at short time period was not affected by these drugs but was slightly, though significantly ( $p < 0.05$ ), dampened after long time treatment (Fig. 6E-H). Although the drug-induced effects on IgE and IgG1 specific response were not very pronounced in numerical expression (about 2–3 times) they were significant and differed in sign between antibody classes and could not be accidental. This observation supports our hypothesis that dampened B-cell proliferation could shift the balance towards specific IgE rather than IgG1 production.

## **Discussion**

Previously we have shown that in young human individuals allergic to house mite dust allergen specific serum IgE production is not associated with production of other Ig classes [1] and may be triggered in

sites other than SLOs germinal centers, for example in TLSs. In present study, we demonstrate that model antigen OVA at low dose induces specific IgE production only when administrated in TLSs-enriched region, withers in particular. Fat-associated lymphoid clusters (FALCs) are relatively constant structures [39] in comparison with inducible bronchial-associated lymphoid tissues (iBALTs) [24, 39] and, so, immunization in above mentioned region may represent an adequate model of chronic allergen instillation into respiratory tract of patients containing iBALTs. In such patients, iBALTs can be formed due to chronic bacterial or virus infections or even due to inhalation of different particulate matter, though it was shown only in mouse model [24, 40]. As a matter of fact, FALCs are not the same structures as iBALTs and supporting tissues are completely different. While not denying it, we assume that the basic functional principles for these two types of TLSs are generally the same. Due to the presence of ILC2s in FALCs as well as T helper 2 and M2 macrophages [39] the basic mechanisms of IgE production triggering could be the similar both in FALCs and in iBALTs. It should be emphasized that main innate immunity linked pro-allergic cytokines such as IL-33 and TSLP are expressed not only in epithelial barriers but in adipose tissue as well [26, 27].

Low dose of chronically administrated antigen induced more pronounced IgE response. It is very important that in our model the degree of sensitization was linked primarily with specific IgE response but not with IgG1 or IgG2a. Our allergic model is considered as clinically relevant representative reconstruction of allergy progression in humans. It is also interesting that specific IgE and IgG1 levels in this model were relatively weakly interconnected if any relation even occurred. Despite this fact, low antigen doses trigger IgE and IgG1 class switching in the same anatomical location – white adipose tissue. Withers adipose tissue rather than regional lymph nodes was the only site of B-cell activation upon low dose antigen administration. It was probably due to the lack of antigen delivering to SLOs in case of low doses. In the absence of PRR stimulus dendritic cells are either not activated or activated in tolerogenic but not immunogenic way, and their maturation and following migration from tissues to SLOs are rare [41]. Therefore, low antigen doses accumulate only in tissues but not in SLOs.

Low antigen doses are very weak immunological stimulus which does not engage PRRs and very weak triggers of FALCs immune cell activation. So, cells activated by low antigen doses in these structures would be stimulated occasionally in the presence of very low amounts of proliferation supporting stimulus (like IL-2, IL-4, TNF- $\alpha$  etc.) and cell attracting chemokines. The latter may intensify activation-induced B cell migration from tissues to SLOs. This hypothesis was confirmed by reduced expression of *cd19* gene despite induction of genes corresponding to B-cell activation and Ig class switching.

Similarly, the data on *postswitch*  $\epsilon$  and  $\gamma 1$  transcripts expression indicates that despite the local tissue-restricted B-cell activation IgE production occurs mostly in SLOs, i.e. regional lymph nodes. It is low rather than high antigen doses administration that induced high levels of specific IgE production, according to ELISA, and enhanced expression of *postswitch*  $\epsilon$  transcripts in murine lymph nodes. Also the expression of *postswitch*  $\epsilon$  transcripts in lymph nodes normalized to *Gapdh* was relatively weak which probably corresponded to larger numbers of mature antibody producing cells. This reflects the presence and availability of the more numbers of B-cell niches in lymph nodes than in withers. In mice, upon high dose

antigen administration, the migration of IgE-switched B-cells from TLSs to SLOs does not occur or occurs relatively weakly, therefore, the IgE switched B-cells that not completely differentiate to plasma cells accumulate in TLSs. These cells are probably responsible for low level IgE production observed in high dose group.

Supposing that (a) in TLSs, namely FALCs, B-cell proliferation, especially in response to weak immunological stimulus, is hampered due to low numbers of B-cell supporting niches and (b) it is FALCs B-cells that is switched to IgE antibodies in response to low antigen doses, it is logical to speculate that hampered proliferation in FALCs is responsible for shifting the balance towards IgE production. Indeed, in this work, we shown that anti-proliferative drugs, especially hydrophobic (and apparently locally acting) etoposide, enhance specific IgE production. But the effects of the same drugs on IgG1 production were opposite. These data are in agreement with recent observations. For example, in reporter transgenic mice expressing IgE fused with fluorescent protein, the isotype switching occurs mostly in early stages of anti-helminthic immune response when B-cells proliferation in germinal centers is not so rapid as at the later stages [17, 18]. IgE<sup>+</sup> B-cells have diminished expression of anti-apoptotic proliferation supporting transcription factor Bcl6 [17]. In the other hand, recently switched IgE<sup>+</sup> B-cells have low levels of costimulatory molecules expression and were more apoptosis prone than IgG1<sup>+</sup> B-cells [18].

In conclusion, in our research work we introduced a new low-dose reproducible mouse allergy model which can be an alternative to standard allergy models with high antigen dose usage. In this new model all mice produce significant specific IgE levels and IgE isotype switching is triggered in damaged tissue but not in lymph nodes. So this new model may be more refinement and clinically relevant that commonly used high antigen dose and adjuvant based mouse allergy models. We hope that this model may be interesting and useful for further investigations of IgE production triggering and development.

## Conclusions

In summary, low dose of administrated antigen triggers B-cell activation and IgE class switching exclusively in TLSs in the damaged tissue. High antigen doses weakly activate B-cells in TLSs and weakly, if any (dependent on anatomical site), induce IgE antibody production. Hampered B-cell proliferation could shift the balance from IgG1 towards IgE switching in these structures. Despite this fact mature IgE producing plasma cells differentiate from immature precursors in SLOs where they migrate after activation in TLSs.

## Methods

### Mice

Female wild type BALB/c mice (6–8 weeks old) were obtained from Andreevka Center (Stolbovaya, Russian Federation). Mice were housed in SPF conditions about 2 weeks before experiments. During all experimental procedures mouse were kept in 12-hour light dark cycle at room temperature in a special

room in vivarium. Mice were housed in plastic cages (10–12 mice for 1 cage) with filings as a bedding material. Mice were fed *ad libitum* by granular feed. Mice were treated and their health status was monitored according to an approved by IBCh RAS IACUC protocol.

## Immunization and blood sampling

Ovalbumin (OVA, Sigma Aldrich, Darmstadt, Germany) was used as a model antigen. Mice received OVA by intraperitoneal (i.p.) and subcutaneous injections in foot pad (f.p.) or in the withers areas (W). OVA was administrated repeatedly 3 times a week for 4–5 weeks (total 14 immunizations) in low (100 ng/injection) or high (10 µg/injection) doses in total volume of 100 µl saline. Before study the randomization procedure was performed. Two control groups were commonly used in the study, namely intact mice and saline immunized mice (n = 5) We used 2 experimental groups for each immunization protocol when studied the dependence of antibody production from the site of immunization and antigen dose (n = 5). The number of animals in groups was determined so that for U-test quantification  $p < 0,01$  values could be obtained. Blood sampling from retro-orbital sinus was performed after 7th (17th day) or 14th (33th day) of immunization. Blood was collected, coagulated for 15 minutes at 37°C and spinned (600 g, 20 min). Serum was collected and stored at -20°C prior to use.

In some experiments, mice (n = 5) were immunized with low OVA dose with anti-proliferative drugs doxorubicin and etoposide (Sigma Aldrich) in doses of 2 and 20 µg/ml which corresponded to doses 0.1 and 1 mg/kg. These doses are too low in comparison with usually used for treatment of malignancies [42, 43] and very unlikely cause systematic harmful effects. Doxorubicin was administrated in saline and etoposide in the vehicle containing 0,25% DMSO + 10% PEG-400 because of water insolubility. Two control groups were used, i.e. saline immunized mice and mice immunized with antigen alone. Two experimental groups for each drug (with different drug dose) were used (n = 6–7).

## ELISA

Microtiter plates (MaxiSorb®, Costar, USA) were coated with 50 µl of 5 µg/ml OVA solution in PBS (pH = 7.2) and incubated overnight at + 4°C. Following extensive washing with PBS containing 0.05% Tween-20 (PBS-T) and subsequent blocking with 5% BSA in PBS, plates were incubated with serially diluted serum samples at + 4°C overnight. Next day plates were subsequently exposed to primary biotin-labeled anti-mouse antibodies (BioLegend, San Diego, CA, USA ) to either IgEa (clone UH297) or IgG1 (clone RMG1 1) or IgG2a (clone RMG2a 62), and streptavidin-conjugated HRP. Plates were further processed with substrate solution (3,3',5,5'-tetramethylbenzidine) Optical densities (OD) were measured with automatic 96-well plate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm with subtraction of optical density at 620 nm that does not correspond to the reaction colored product. Antibody quantities were estimated as OVA-specific antibody titers defined as the serum dilution, in accordance according to 4-PL regression analysis.

For estimation of total IgE levels, plates were coated with unlabeled anti-mouse IgE (clone RME1, BioLegend). Serum samples were added in 1:100 dilution in duplicates with different dilutions of mouse

IgE  $\kappa$  (MEA-36, BioLegend). In following steps biotin labeled anti-mouse IgE (UH297) and streptavidin-HRP were added subsequently as described above.

## Systematic anaphylactic response measurement

For measurement of systematic anaphylactic response in mice after full immunization protocol, OVA in challenging dose (0.2 ml of 2.5 mg/ml solution) was administrated intraperitoneally to animals. Body temperature was measured with infrared thermometer CEM DT-8806S, (SEM Test Instruments, Moscow, Russia) as it was performed in [44] before OVA administration and 45 minutes after. The difference of temperature between two time points was defined. The experiment was performed in laboratory.

## Local anaphylactic response measurement

Standard Evans Blue based test was used to measure local anaphylaxis response. Briefly, abdominal region of each mouse was shaved and after that 10  $\mu$ l of 1 mg/ml OVA were administrated subcutaneously in this area. After 3 minutes 200  $\mu$ l of 0.5% Evans Blue solution were administrated in the tail vein. After 15 minutes reaction intensity was evaluated. The experiment was performed in laboratory.

## Gene expression measurement by qPCR

After 14th immunization mice were sacrificed in laboratory by cervical dislocation. Lymph nodes were harvested, homogenized and cells were spinned in PBS (600 g, 10 min), followed by the RNA extraction using phenol chloroform based extraction protocol. (ExtractRNA, Evrogen, Moscow, Russia). In the case of white adipose tissue small pieces were harvested and washed from blood in the PBS and then subjected to RNA extraction with the same reagent. cDNA was prepared from the samples with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed with SybrGreen I based kits from BioLabMix (Novosibirsk, Russia). Expression of target genes was estimated by normalizing to expression of house-keeping gene GAPDH and was calculated as  $2^{-d(dCt)}$  in comparison with expression in the tissues of intact control mouse. In the case of postswitch  $\epsilon$  transcripts for analysis of absolute IgE-producing B cell numbers in samples, normalization to GAPDH was performed. For measurement of IgE-producing B cell content in B cell fraction, normalization to CD19 was performed. Primers were designed and synthesized by Evrogen. The following primers were used:

for GAPDH F: GGTGCTGAGTATGTCGTGGA; R: TGGAAGAGTGGGAGTTGCTG;

IL-25 F: CCCAGCAAAGAGCAAGAACC; R: ATCCTCTAGCAGCACAAGCG;

IL-33 F: GTCTCCTGCCTCCCTGAGTA; R: GTGGTGCCTGCTCTTCTGAA;

TSLP F: CTGCCTGAATCAAACCTCACAA; R: TGACTGCCCGAACTGTCATT;

CD19 F: TTCACTACTGAGCCTAAGCCTTG; R: CAACAGCCAGAGCCACACT;

AICDA F: ACAGCACTGAAGCAGCCTT; R: GCCCAGCGGACATTTTTGAA;

Bcl6 F: CAGTCCCCACAGCATACAGA; R: CCTCAGAGAAACGGCAGTCA;

EBI2 F: CATAAAAGGACGCCTGCTCG; R: TTGCCAGTGGGGTAGTGAAA;

germline  $\epsilon$  F: GCACAGGGGGCAGAAGAT; R: CCAGGTCACAGTCACAGGAT;

germline  $\gamma$ 1 F: CACGGGAGATTGGGAAGGAG; R: TTTGGGCAGCAGATCCAGG;

Gata3 F: TGGAGGAGGAACGCTAATGG; R: GATGTGGCTCAGGGATGACA;

NMUR1 F: ATGACTCCTCCCTGCCTCA; R: GAGCACCAGCATATCGGACA;

postswitch  $\epsilon$  F: CAGGCTGCTGCTGGGTAG; R: GCTGGTGGTGACCTTGAGTT;

postswitch  $\gamma$ 1 F: ACATGCTCTGTGTGAACTCCC; R: AGGTCAGACTCCAGGACAGC. Ct values were determined for each sample. Reaction was performed in DT Prime Amplificator (DNA Technology, Moscow, Russia ) according to the following protocol: initial denaturation at + 95°C for 3 minute and then 50 cycles with: 15 s denaturation at + 95°C, 45 s annealing and elongation at + 60°C.

## Statistics

For comparisons between experimental groups and samples, nonparametric Mann-Whitney test was used. Levels of  $p < 0.05$  were considered statistically significant. For correlation coefficients determination, Spearman test was used. The data from each individual animal was considered as a single point. Mean and standard deviations for each compared group were calculated.

## Abbreviations

CFA – Complete Freund’s Adjuvant

ELISA – Enzyme Linked Immunosorbent Assay

FALCs – Fat associated lymphoid clusters

f.p. – foot pad

iBALT – Inducible bronchial-associated lymphoid clusters

ILC2 - Innate type 2 lymphoid cells

i.p. - intraperitoneal

LPS - Lipopolysaccharide

OVA - Ovalbumin

s.c. - subcutaneous

## Declarations

### Ethics approval and consent to participate

This study includes data with laboratory animals. All animal experiments were carried out according to Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry IACUC. This study does not contain human participants and any data obtained on human tissue samples.

### Consent for publication

Not applicable.

### Availability of data and materials

Not applicable

### Competing interests

The authors declare that they have no competing interests

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### Author contribution

D.B.C. and D.S.T. immunized mice and performed ELISA. D.B.C. and D.Yu. R. extracted and purified RNA samples from mice tissue samples and carried out reverse transcription and quantitative PCR reactions. O.D.K. and G.V.F. performed statistic data processing and data analysis. O.D.K. and G.V.F. were blinded when performed data analysis because they do not know the immunization protocol and availability or lack of usage of anti-proliferative drugs for groups. D.B.C. and G.V.F. wrote manuscript. All authors have read and approved the final manuscript.

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## References

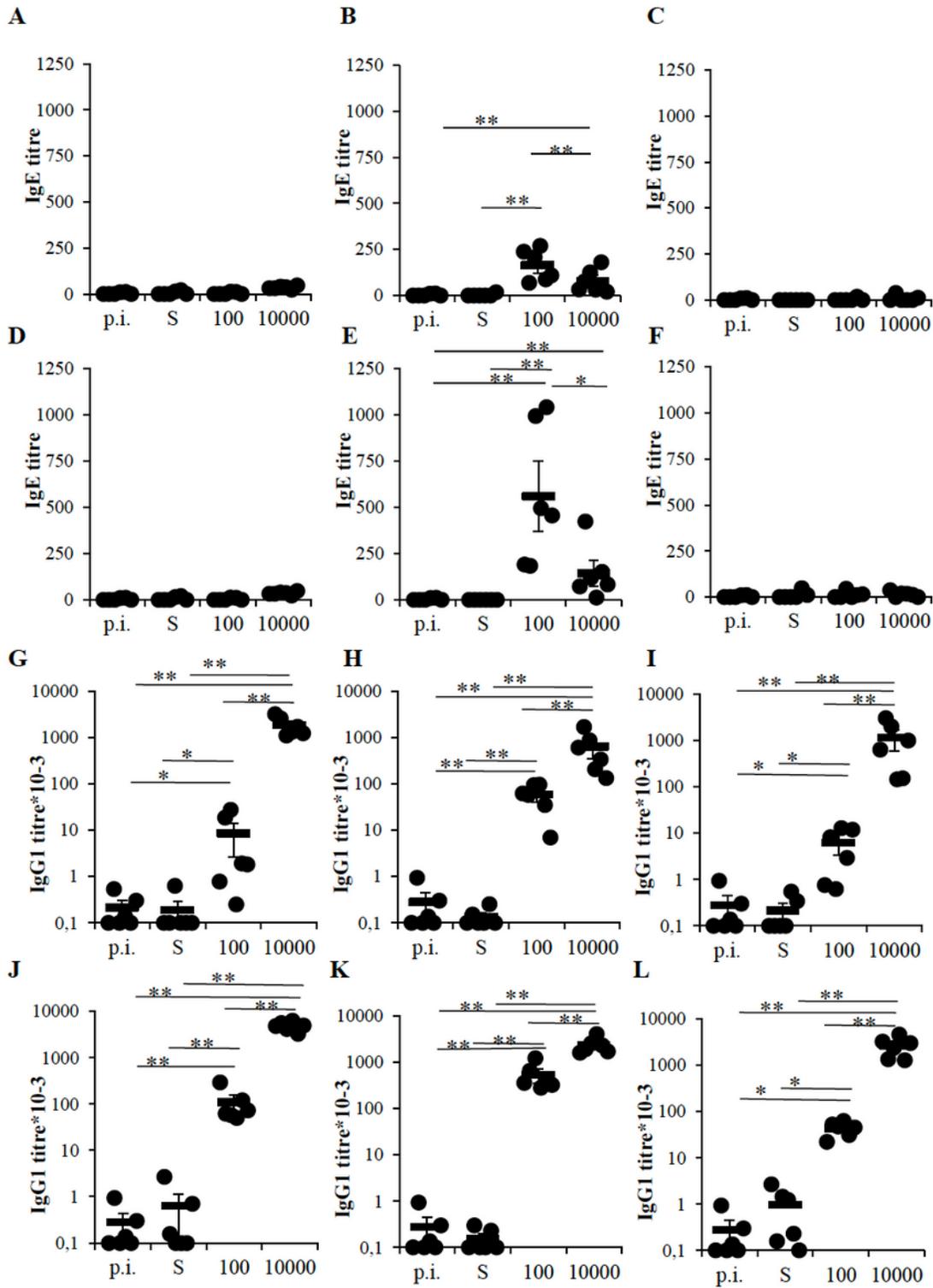
1. Svirshchevskaya E, Fattakhova G, Khlgatian S, Chudakov D, Kashirina E, Ryazantsev D, et al. Direct versus sequential immunoglobulin switch in allergy and antiviral responses. *Clin Immunol.* 2016;170:31–8.
2. Licona-Limon P, Kim LK, Palm NW, Flavell RA. Th2, allergy and group 2 innate lymphoid cells. *Nat Immunol.* 2013;14(6):536–42.
3. Mjosberg J, Bernink J, Golebski K, Karrich JJ, Peters CP, Blom B, et al. The transcription factor GATA3 is essential for the function of human type 2 innate lymphoid cells. *Immunity.* 2012;37:649–59.
4. Hansen JS, Larsen ST, Poulsen SK, Nielsen GD. Adjuvant effects of inhaled mono-2-ethylhexyl phthalate in BALB/cJ mice. *Toxicology.* 2007;232(1–2):79–88.
5. Clausen SK, Bergqvist M, Poulsen LK, Poulsen OM, Nielsen GD. Development of sensitisation or tolerance following repeated OVA inhalation in BALB/cJ mice. Dose-dependency and modulation by the Al(OH)<sub>3</sub> adjuvant. *Toxicology.* 2003;184(1):51–68.
6. Kim YH, Yoshimoto M, Nakayama K, Tanino S, Fujimura Y, Yamada K, Tachibana H. Tannic acid, a higher galloylated pentagalloylglucose, suppresses antigen-specific IgE production by inhibiting  $\kappa$  germline transcription induced by STAT6 activation. *FEBS Open Biol.* 2013;3:341–5.
7. van der Ventel ML, Nieuwenhuizen NE, Kirstein F, Hikuam C, Jeebhay MF, Swoboda J, et al. Differential responses to natural and recombinant allergens in a murine model of fish allergy. *Mol Immunol.* 2011;48(4):637–46.
8. Chang Y-S, Kim Y-K, Jeon SG, Kim S-H, Kim S-S, Park HW, et al. Influence of the Adjuvants and Genetic Background on the Asthma Model Using Recombinant Der f 2 in Mice. *Immune Netw.* 2013;13(6):295–300.

9. Samuelsen M, Nygaard UC, Lovik M. Allergy adjuvant effect of particles from wood smoke and road traffic. *Toxicology*. 2008;246(2–3):124–31.
10. Niederberger V, Niggemann B, Kraft D, Spitzauer S, Valenta R. Evolution of IgM, IgE and IgG(1–4) antibody responses in early childhood monitored with recombinant allergen components: implications for class switch mechanisms. *Eur J Immunol*. 2002;32(2):576–84.
11. Resch Y, Michel S, Kabesch M, Lupineck C, Valenta R, Vrtala S. Different IgE recognition of mite allergen components in asthmatic and non-asthmatic children. *J Allergy Clin Immunol*. 2015;136(4):1083–91.
12. Pereira FA, Silva DA, Cunha-Junior JP, Almeida KC, Alves R, Sung SJ, Taketomi EA, IgE, IgG<sub>1</sub>, and IgG<sub>4</sub> antibody responses to *Blomia tropicalis* in atopic patients. *Allergy*. 2005;60(3):401–6.
13. Miranda DO, Silva DA, Fernandes JF, Queiros MJ, Chiba CF, Ynoue LH, et al. Serum and salivary IgE, IgA, and IgG<sub>4</sub> antibodies to *Dermatophagoides pteronyssinus* and its major allergens, Der p1 and Der p2, in allergic and nonallergic children. *Clin Dev Immunol*. 2011;2011:302739.
14. Ecki-Dorna J, Villazala-Merino S, Linhart B, Karaulov AV, Zhernov Y, Khaitov, et al. Allergen-Specific Antibodies Regulate Secondary Allergen-Specific Immune Responses. *Front Immunol*. 2019;17(9):3131.
15. Aalberse RC, Lupineck C, Siroux V, Nadif R, Just J, Bousquet J, et al. sIgE and sIgG to airborne atopic allergens: Coupled rather than inversely related responses. *Allergy*. 2018;73(11):2239–42.
16. Aalberse RC, Platts-Mills TA, Rispens T. The Developmental History of IgE and IgG<sub>4</sub> Antibodies in Relation to Atopy, Eosinophilic Esophagitis, and the Modified Th2 Response. *Curr Allergy Asthma Rep*. 2016;16(6):46.
17. Talay O, Yan D, Brightbill HG, Straney EM, Zhou M, Ladi E, et al. IgE + memory B cells and plasma cells generated through a germinal-center pathway. *Nat Immunol*. 2012;13(4):396–404.
18. Yang Z, Sullivan BM, Allen C. Fluorescent In Vivo Detection Reveals that IgE + B Cells Are Restrained by an Intrinsic Cell Fate Predisposition. *Immunity*. 2012;36:857–72.
19. Fiset PO, Cameron L, Hamid Q. Local isotype switching to IgE in airway mucosa. *J Allergy Clin Immunol*. 2005;116(1):233–6.
20. Gevaert P, Nouri-Aria KT, Wu H, Harper CE, Takhar P, Fear DJ, et al. Local receptor revision and class switching to IgE in chronic rhinosinusitis with nasal polyps. *Allergy*. 2013;68(1):55–63.
21. Gona-Hopler M, Pfaller B, Argeny J, Kanolzer S, Gruber S, Schmidthaler S, Renner S. *Aspergillus fumigatus*-specific immunoglobulin levels in BALF of CF patients. *ERJ Open Res*. 2017;3(4):00067.
22. Yadava K, Bollyky P, Lawson LA. The formation and function of tertiary lymphoid follicles in chronic pulmonary inflammation. *J Immunol*. 2016;149(3):262–9.
23. Hwang JY, Rendall TD, Silva-Sanchez A. Inducible Bronchus-Associated Lymphoid Tissue: Taming Inflammation in the Lung. *Front Immunol*. 2016;7:258.
24. Foo SY, Zhang V, Lalwani A, Lynch JP, Zhuang A, Lam, et al. Regulatory T cells prevent inducible BALT formation by dampening neutrophilic inflammation. *J Immunol*. 2015;194(9):4567–76.

25. Schoetti T, Fisher IP, Ussar S. Heterogeneity of adipose tissue in development and metabolic function. *J Exp Biol.* 2018;7:221. (Pt Suppl 1).
26. Zeida M, Wernly B, Demyanets S, Kaun C, Hammerle M, Schranz M, et al. Severe obesity increases adipose tissue expression of interleukin-33 and its receptor ST2, both predominantly detectable in endothelial cells of human adipose tissue. *Int J Obes (Lond).* 2013;37(5):658–65.
27. Turcot V, Bouchard L, Faucher G, Garneau V, Tchernof A, Deshaies Y, et al. Thymic stromal lymphopoietin: an immune cytokine gene associated with the metabolic syndrome and blood pressure in severe obesity. *Clin Sci (Lond).* 2012;123(2):99–109.
28. Moro K, Yamada T, Tanabe M, Takeuchi T, Ikawa T, Kawamoto K, et al. Innate production of Th2 cytokines by adipose tissue-associated c-Kit1Sca-11 lymphoid cells. *Nature.* 2010;463:540–46.
29. Molofski AB, Nussbaum JC, Liang H-E, van Dyken SJ, Cheng LE, Mohapatra A, et al. Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J Exp Med.* 2013;210(3):535–49.
30. Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity.* 2008;28(4):581–9.
31. Beutier H, Gillis CM, Iannascoli B, Godon O, England P, Sibilano R, et al. IgG subclasses determine pathways of anaphylaxis in mice. *J Allergy Clin Immunol.* 2017;139(1):269–80.e7.
32. Martin RK, Damle SR, Valentine EA, Zeliner MP, James BN, Lownik JC, et al. B1 cell IgE impedes mast cell-mediated enhancement of parasite expulsion through B2 IgE blockade. *Cell Rep.* 2018;22(7):1824–34.
33. Enoksson SL, Grasset EK, Hagglot T, Mattsson N, Keiser Y, Gabrielsson S, et al. The inflammatory cytokine IL-18 induces self-reactive innate antibody responses regulated by natural killer T cells. *Proc. Natl. Acad. Sci. U.S.A.* 2011;108(51):E1399-1407.
34. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell.* 2000;102(5):553–63.
35. Kanhere A, Hertweck A, Bhatia U, Gokmen MR, Perucha E, Jackson I, et al. T-bet and GATA3 orchestrate Th1 and Th2 differentiation through lineage-specific targeting of distal regulatory elements. *Nat Commun.* 2012;3:1268.
36. Cardoso V, Chesne J, Ribeiro H, Garsia-Cassani B, Carvalho T, Bouchery T, et al. Neuronal regulation of type 2 innate lymphoid cells via neuromedin U. *Nature.* 2017;549(7671):277–81.
37. Schwartz-Cornil I, Epardaud M, Albert JP, Bourgeois C, Geratd F, Raoult I, Bonneau M. Probing leukocyte traffic in lymph from oro-nasal mucosae by cervical catheterization in a sheep model. *J Immunol Methods.* 2005;305(2):152–61.
38. Rayamajhi M, Delgado C, Condon TV, Riches DW, Lenz LL. Lung B cells promote early pathogen dissemination and hasten death from inhalation anthrax. *Mucosal Immunol.* 2012;5(4):444–54.

39. Cruz-Migoni S, Caamano J. Fat-Associated Lymphoid Clusters in Inflammation and Immunity. *Front Immunol.* 2016;7:612.
40. Kuroda E, Ozasa K, Temizoz B, Ohata K, Koo CX, Kanuma T, et al. Inhaled fine particles induce alveolar macrophage death and interleukin-1a release to promote inducible bronchus-associated lymphoid tissue formation. *Immunity.* 2016;45:1299–310.
41. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J.* 2014;33(10):1104–16.
42. Zocchi E, Tonetti M, Polvani C, Guida L, Benatti U, De Flora A. Encapsulation of doxorubicin in liver-targeted erythrocytes increases the therapeutic index of the drug in a murine metastatic model. *Proc. Natl. Acad. Sci. U.S.A.* 1989;86(6):2040–2044.
43. Lee JS, Takahashi T, Hagiwara A, Yoneyama C, Itoh M, Sasabe T, et al. Safety and efficacy of intraperitoneal injection of etoposide in oil suspension in mice with peritoneal carcinomatosis. *Cancer Chemother Pharmacol.* 1995;36(3):211–6.
44. Kawakami Y, Sielski R, Kawakami T. Mouse body temperature measurement using infrared thermometer during passive systemic anaphylaxis and food allergy evaluation. *J Vis Exp* 2018;139:DOI:10.3791/58391.

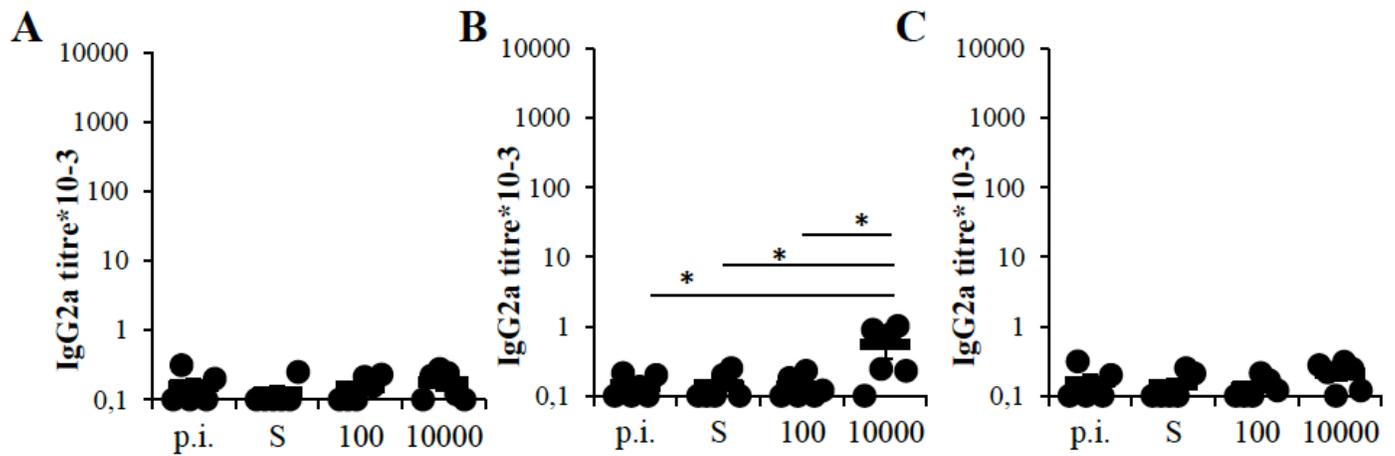
## Figures



**Figure 1**

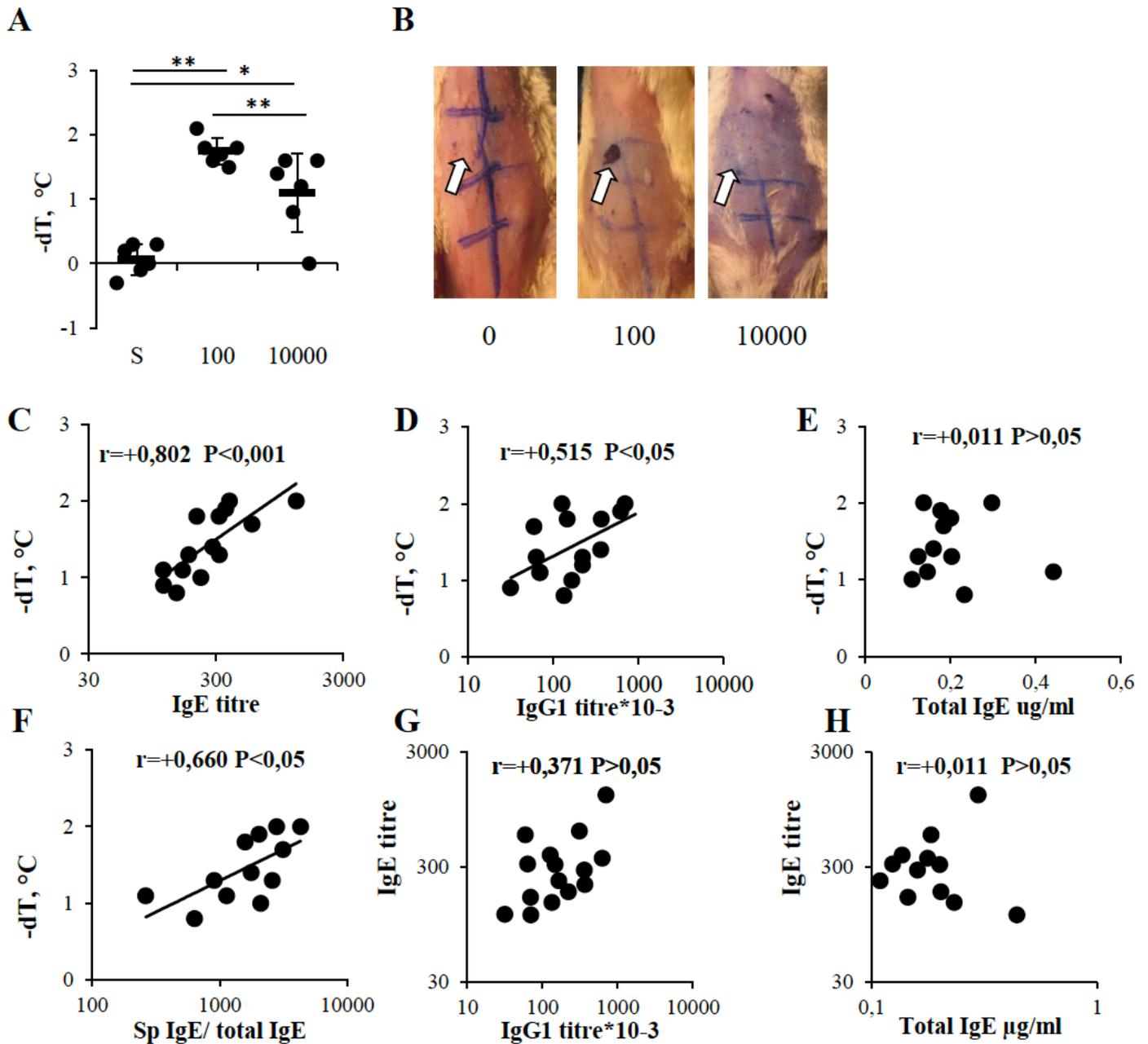
Specific IgE, but not IgG1 production, is induced mostly by low antigen doses, and is depends on the immunization site in BALB/c mice. Specific IgE (A-F) and IgG1 (G-L) production in different immunization groups (total number of mice 45): pre-immune (p.i.), immunized with saline (S), 100 ng (100) or 10 µg (10000) OVA intraperitoneally (15/45 mice) (A, D, G, J), subcutaneously in withers (B, E, H, K) or foot pad (10000) OVA intraperitoneally (15/45 mice) (A, D, G, J), subcutaneously in withers (B, E, H, K) or foot pad (C, F, I, L) upon 7th (17th day) (A-C, G-I) and 14th (33th day) immunizations (D-F, J-L). Data represents

mean values  $\pm$  SD from three independent experiments, (n=5 mice per group) Mann-Whitney test was used for p value estimation.\* - p<0.05; \*\* - p<0.01. There was no any adverse effects in either experimental groups.



**Figure 2**

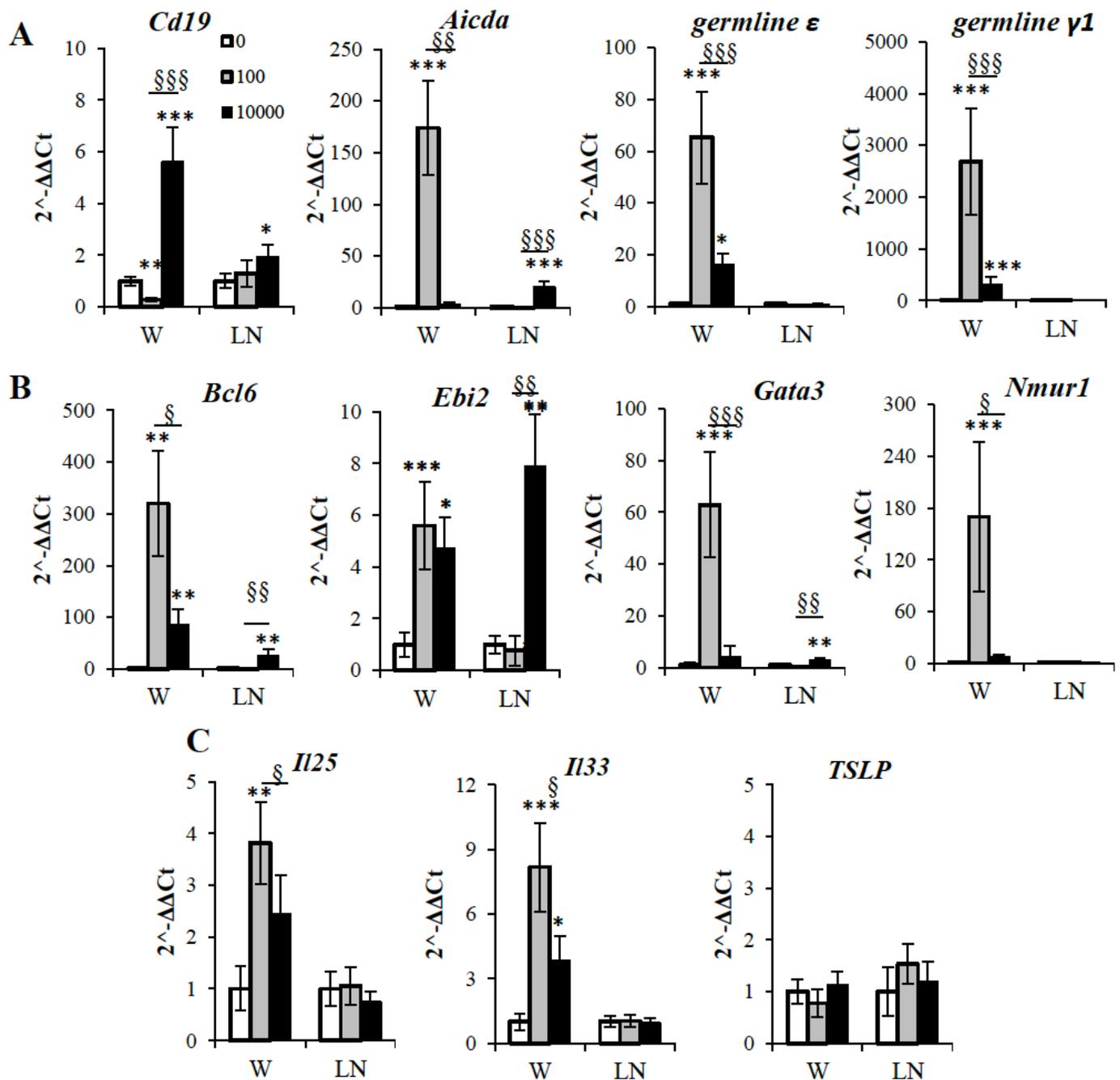
Specific murine IgG2a production is not induced during long-term low dose immunization regardless administration route. Specific IgG2a production in several immunization groups (total number of mice 45): pre-immune (p.i.), immunized with saline (S), 100ng (100) or 10 µg (10000) OVA intraperitoneally (A), subcutaneously in withers (B) or foot pad (C) upon 14th immunization. Data are representative from 3 independent experiments (n=5 mice per group). Error bars are standard deviation. \* - p<0.05. There was no any adverse effects in either experimental groups



**Figure 3**

Systematic and local anaphylactic response in BALB/c mice is associated mostly with specific IgE production, and much less, with specific IgG1 production. Temperature change (shown with opposite sign) after systematic allergen challenge in different immunization groups: saline, 100 ng (100) or 10 µg (10000) upon 14th immunization (A) (total number of mice 18, 6 mice for each group). Representative images of Evans Blue local anaphylaxis assay in different immunization groups of the same mice. Data are representative from 3 independent experiments (n=6 mice per group). Error bars are standard deviation. (B). Correlation between specific IgE (C), IgG1 (D), total IgE (E) production and temperature change after systematic allergen challenge in low dose (100 ng) immunized mice (total number of mice 14). Correlations between Specific to total IgE ration and temperature changes (F) (total number of mice

14). Correlations between specific IgG1 and specific IgE production (G) and between specific and total IgE (H) (total number of mice 14). There was no any adverse effects in either experimental groups



**Figure 4**

B-cell activation in low-dose immunization protocol occurs in withers adipose tissue, but not in lymph nodes. Expression of B-cell specific gene *cd19* and genes associated with B-cell antibody class switching and normalized to control intact murine samples (0) in low-dose (100) and high-dose (10000) immunization groups after 14th immunizations in withers adipose tissue (W) or in regional lymph nodes (LN) (A); expression of genes linked with immune cell activation (B); expression of innate-immunity linked

pro-allergic cytokines (C) in the same samples. Data are representative from 3 independent experiments (n=5 mice per group, total number of mice 15). Error bars are standard deviation. Statistical significance between immunized and intact mouse groups: \* - p<0.05; \*\* - p<0.01; \*\*\* - p<0.001; between low and high dose mouse groups: § - p<0.05; §§ - p<0.01; §§§ - p<0.001. There was no any adverse effects in either experimental groups

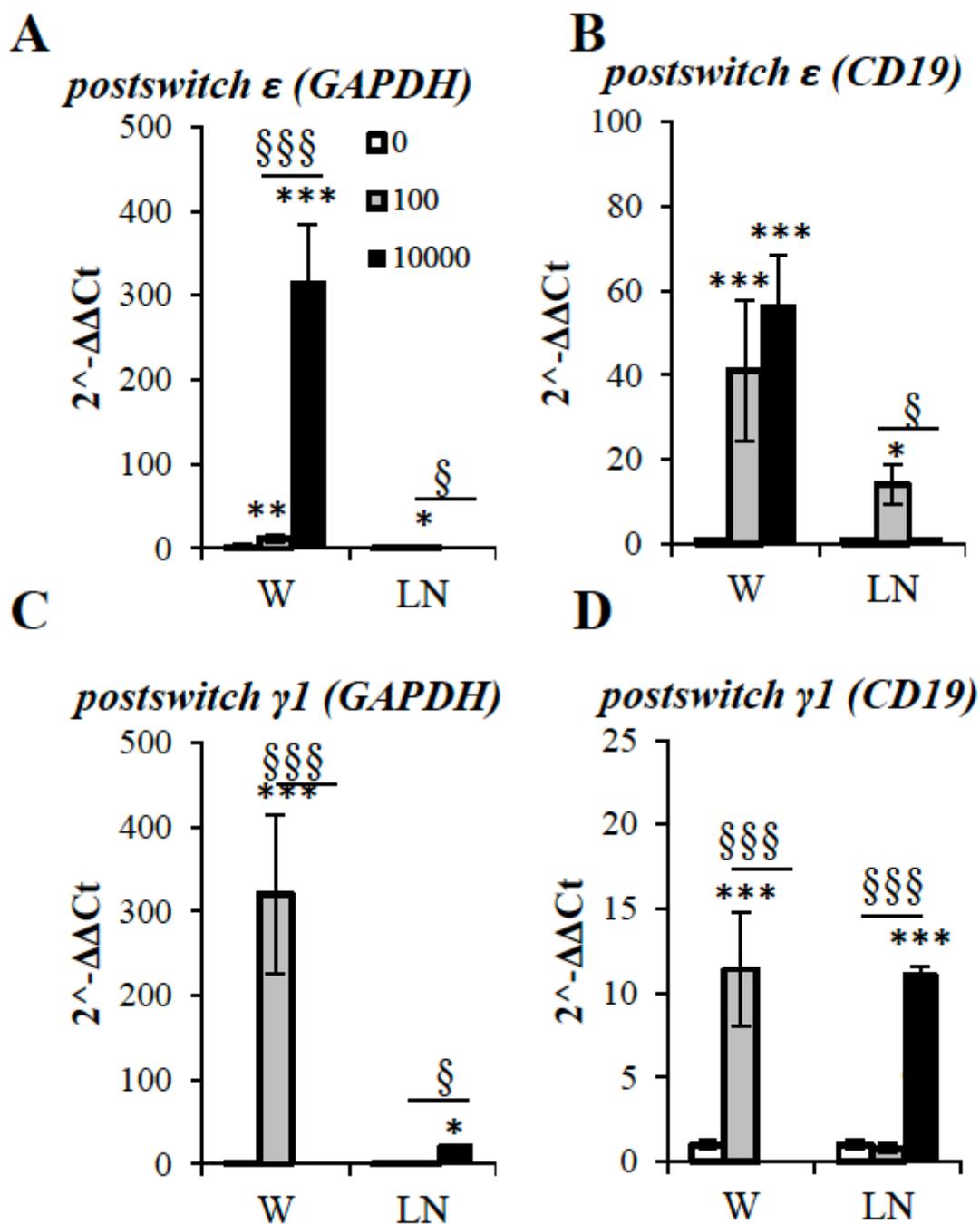
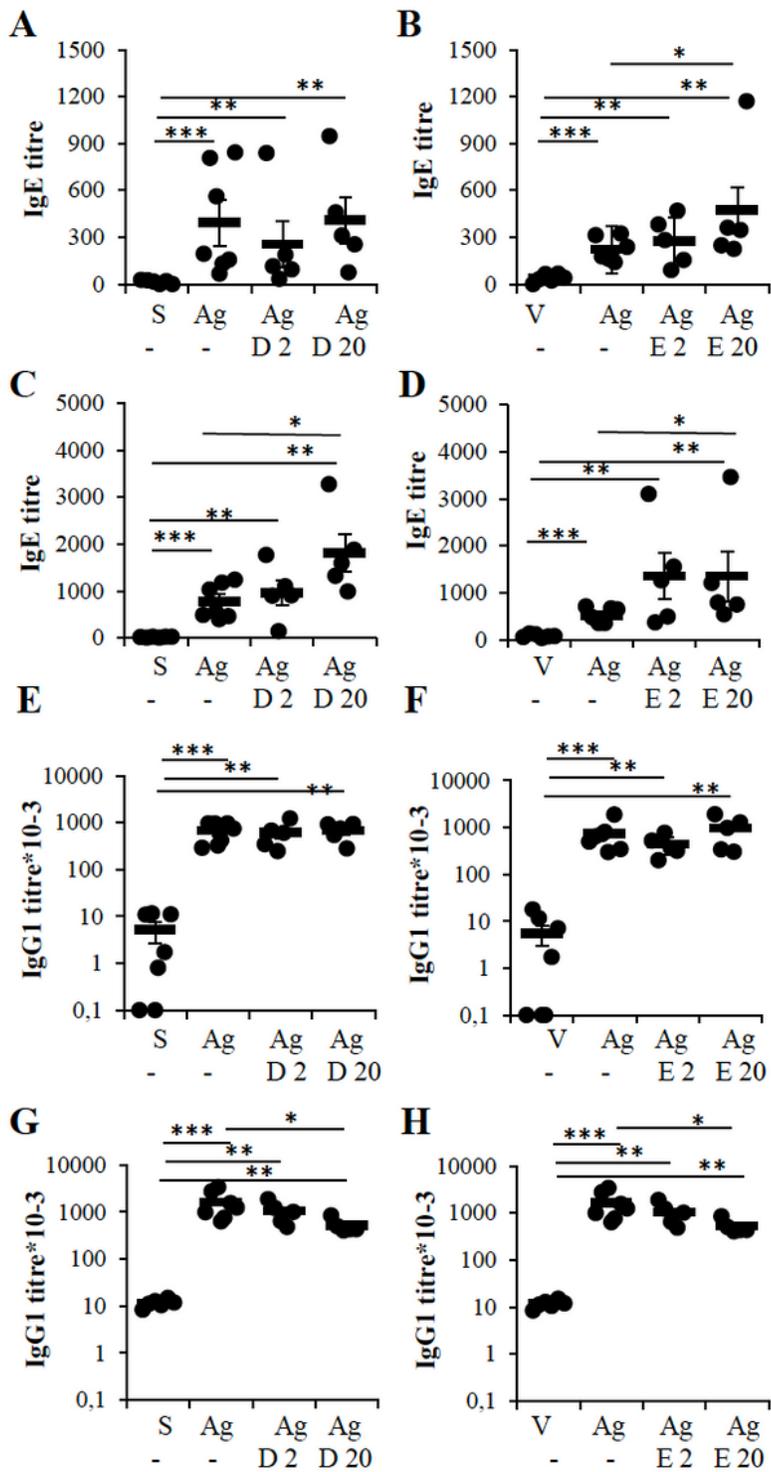


Figure 5

Upon allergen challenge, a portion of antigen-activated B-cells migrates to SLOs where specific IgE production primarily occurs. Expression of postswitch  $\epsilon$  (A-B) or postswitch  $\gamma 1$  (C-D) transcripts normalized to GAPDH (A, C) or CD19 (B, D) in the withers (W) and regional lymph nodes (LN) of intact (0), low-dose (100) and high dose (10000) immunized mice. Data are representative from 3 independent experiments (n=5 mice per group, total number of mice 15). Error bars are standard deviation. Statistics significance between immunized and intact mouse groups: \* -  $p < 0.05$ ; \*\* -  $p < 0.01$ ; \*\*\* -  $p < 0.001$ ; between low and high dose mouse groups: § -  $p < 0.05$ ; §§ -  $p < 0.01$ ; §§§ -  $p < 0.001$ . There was no any adverse effects in either experimental groups



**Figure 6**

Water soluble and lipophilic anti-proliferative drugs stabilize and enhance specific IgE rather than IgG1 production. Specific IgE (A-D) and IgG1 (E-H) antibody production in mice, immunized with saline (S), 100 ng OVA alone (Ag) or in the presence of 2 µg/dose or 20 µg/dose of Doxorubicin (Ag+D 2 and Ag+D 20) (A, C, E, G) or same doses of Etoposide (Ag+E 2 and Ag+E 20) (B, D, F, H) after short-term (7 immunizations) (A-B, E-F) and long-term (14 immunizations) (C-D, G-H) protocols. Total number of mice

23, 6 mice in saline and 7 in antigen control group and 5 mice in each test group. There was no any adverse effects in either experimental groups.

## Supplementary Files

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