

B-cell subsets are modulated during allergic airway inflammation but are not required for the development of respiratory tolerance in a murine model

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Abbreviations: **AHR:** airway hyperreactivity, **Alum:** aluminium hydroxide and magnesium hydroxide, **ASC:** antibody secreting cells, **AUC:** area under the curve, **BALF:** bronchoalveolar lavage fluid, **BLN:** bronchial lymph nodes, **BM:** bone marrow, **Breg cells:** B-cells with a regulatory function, **DCs:**

dendritic cells, **E**: elastance, **FcεRII**: receptor II for the Fc region of immunoglobulin E, **FM**: follicular mature, **FMO**: Fluorescence minus one control, **GC**: germinal center, **H&E**: haematoxylin and eosin, **i.n.**: intranasal, **LPS**: lipopolysaccharide, **MCh**: methacholine, acetyl-β-methylcholine chloride, **MFI**: mean fluorescence intensity, **μMT**: B6.129S2-Igh-6-tm1cgn/J, B-cell deficient mice, **MZ**: marginal zone, **MZp**: marginal zone precursor, **NaCl**: sodium chloride, **OVA**: Ovalbumin, **PAS**: periodic acid-Schiff, **PNA**: peanut agglutinin, **R**: resistance, **Th2**: T helper type 2, **Treg cells**: regulatory T-cells, **TOL**: respiratory tolerance, **T1**: transitional 1, **T2**: transitional 2, **WT**: wildtype.

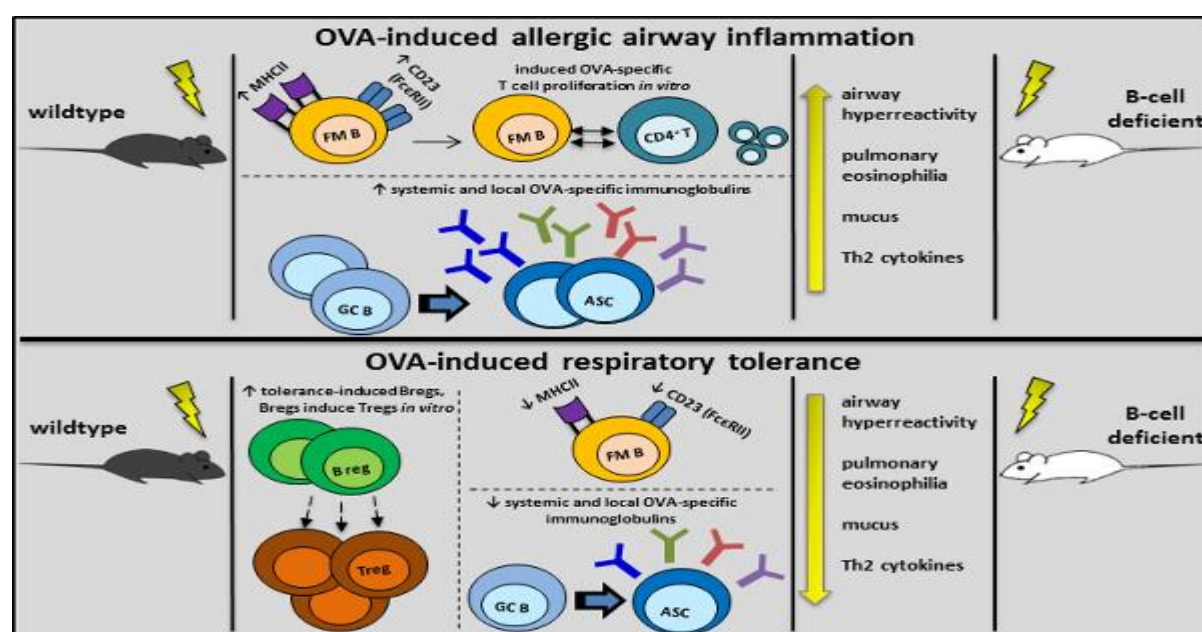
Abstract

Allergic asthma is a widespread chronic inflammatory disease of the airways. The role of different B-cell subsets in developing asthma and respiratory tolerance is not well-known. Especially regulatory B (Breg) cells are proposed to be important in asthma regulation. Using wildtype (WT) and B-cell-deficient (μMT) mice we investigated how B-cells are affected by induction of allergic airway inflammation and respiratory tolerance and whether they are necessary to develop these conditions. WT mice with an asthma-like phenotype, characterized by increased airway hyperreactivity, eosinophilic airway inflammation, mucus hypersecretion and elevated Th2 cytokines, exhibited increased MHCII and CD23 expression on follicular mature B-cells in lung, bronchial lymph nodes (bLN) and spleen, which contributed to allergen-specific T-cell proliferation *in vitro*. Germinal center B-cell numbers were elevated and associated with increased production of allergen-specific

immunoglobulins especially in bLN. In contrast, respiratory tolerance clearly attenuated these B-cell alterations and directly enhanced marginal zone precursor B-cells, which induced regulatory T-cells *in vitro*. However, μ MT mice developed asthma-like and tolerized phenotypes like WT mice.

Our data indicate that although B-cell subsets are affected by asthma-like and respiratory tolerant phenotypes, B-cells are not required for tolerance induction.

OVA-induced allergic airway inflammation (AAI) mobilizes several B-cell subsets to take part in antigen presentation, T-cell proliferation and immunoglobulin production, whereas respiratory tolerance induces Breg cells, Treg cells and diminishes AAI-mediated alterations. Nevertheless, development of both phenotypes does not require B-cells in our mouse model.



Introduction

The incidence of childhood asthma and allergies still rises in western countries since the last century and also the prevalence in Asia, Latin America and Africa increased over the last years [1]. Hence, it is of particular importance to increase our knowledge of specialized cellular players, which mediate immunological tolerance to allergens. Asthma is characterized by airway hyperreactivity (AHR), chronic airway inflammation, mucus hypersecretion, pulmonary infiltration of eosinophils and T helper type 2 (Th2) cells and increased Th2 cytokine production [2-4]. B-cells are most prominent for secretion of allergen-specific immunoglobulins, especially IgE, which provoke asthmatic symptoms [1]. Under nonallergic circumstances antigens mediate antigen-specific tolerance or hyporesponsiveness. Thereby, peripheral T-cell tolerance is important, including suppressive regulatory T (Treg) cells [4]. Moreover, there is a growing body of evidence suggesting that regulatory B (Breg) cells are substantially involved in allergic diseases. Adoptive transfer of activated Breg cells attenuates inflammatory responses in models of contact hypersensitivity [5], allergic airway inflammation [6] and food allergy [7]. Whether B-cells are an essential part of the immunological repertoire that is necessary to establish antigen-specific respiratory tolerance is less known. Additionally, detailed information about the role of immature and mature B-cell subsets in asthma-like and respiratory tolerant phenotypes is missing.

Based on phenotypical, topographic and functional characteristics, several B-cell subpopulations can be identified. Immature, surface-IgM expressing B-cells migrate from the bone marrow (BM) primarily to the spleen [8], where they further develop from early (T1) and late (T2) transitional B-

cells [9] into different mature B-cell stages, namely follicular mature (FM) and marginal zone (MZ) B-cells. Upon antigen encounter, FM B-cells can act as antigen-presenting cells (APCs) and activate T-cells. They enter the germinal center (GC) reaction, receive T-cell help and differentiate into high-affinity and class-switched antibody producing plasma cells or memory B-cells [8]. MZ B-cells are predominantly regarded as innate-like cells which recognize blood-borne antigens [8, 10] but they are also relevant for T-cell-dependent responses [11, 12]. Their direct predecessors, marginal zone precursor (MZp) B-cells [13, 14] have been shown to possess suppressive immune functions and are therefore considered as Breg cells [13, 15].

Here, we investigate which B-cell subpopulations are affected in a murine model of ovalbumin (OVA) induced allergic airway inflammation and respiratory tolerance. Moreover, we sought to determine whether B-cells, especially Breg cells, are needed for the development of respiratory tolerance by using wildtype (WT) and B-cell deficient (μ MT) mice. Our data indicate that several B-cell subsets are affected in both asthma-like and respiratory tolerant phenotype. Nevertheless, B-cells are not required for the development of respiratory tolerance.

Results

Regulatory MZp B-cells induce Treg cells after tolerance induction

There is a growing body of evidence suggesting that B-cells, especially Breg cells, are important regulators in allergic diseases. Therefore, we analyzed if B-cell subsets are modulated by OVA-induced allergic airway inflammation and respiratory tolerance [16-19] (Fig. 1).

Based on CD21 and CD24 expression, CD19⁺ B-cells were separated into T1, T2, FM and MZ/MZp B-cells [20, 21]. The latter were further distinguished into regulatory CD23^{hi} MZp B-cells and CD23^{low} mature MZ B-cells (Fig. 2A) [21]. CD19⁺CD21^{low}CD24^{hi} T1 B-cells were increased in bronchial lymph nodes (bLN) of mice with an asthma-like phenotype, which was completely abrogated by tolerance induction (Fig. 2B). Splenic T1 B-cells showed no differences. CD19⁺CD21^{int}CD24^{hi} T2 B-cells were reduced in allergic and tolerized mice compared to controls (Fig. 2C). In lungs of allergic mice, but not in spleen and bLN, CD19⁺CD21^{int}CD24^{int} FM B-cells were diminished compared to control and tolerized mice (Fig. 2D). Splenic CD19⁺CD21^{hi}CD24^{hi}CD23^{low} MZ B-cells (Fig. 2E) showed no differences between the groups.

Notably, splenic CD19⁺CD21^{hi}CD24^{hi}CD23^{hi} MZp B-cells were increased in allergic mice compared to control and tolerized mice (Fig. 2F). MZp B-cells are considered as IL-10 producing Breg cells. We therefore determined IL-10 production of B-cells in response to lipopolysaccharide (LPS) stimulation. Despite reduced numbers of MZp B-cells in tolerized mice, total CD19⁺ B-cells from allergic and tolerized mice generated similar amounts of IL-10, which was significantly higher compared to B-cells from control mice (Fig. 2G). We next directly tested the capability of MZp B-cells to induce Treg cells, which is one mechanism of Breg cells to exert their regulatory function [22]. For these experiments, MZp B-cells were isolated from mice directly after tolerization (day 0, Fig. 1). Notably, at this early time point, absolute numbers of MZp B-cells are increased in tolerized compared to control mice, suggesting that they are directly induced upon tolerization (Fig. 2H). When MZp B-cells were cultured with OVA-specific CD4⁺ T-cells in the presence of dendritic cells (DCs) and OVA, MZp

B-cells from tolerized mice induced more Foxp3⁺ Treg cells compared to control cells *in vitro* (Fig. 2I, 2J). Taken together, our data show significant modulations of B-cell subsets by induction of allergic airway inflammation, which are abrogated in tolerized mice. Most importantly, tolerized MZp B-cells are able to induce Treg cells.

FM B-cells serve as antigen-presenting cells

In addition to immunoglobulin and cytokine production, B-cells can act as professional APCs. To test their ability for antigen presentation, expression of MHCII molecules was examined. FM B-cells in spleen, bLN and lung of mice with an asthma-like phenotype showed significantly enhanced expression of MHCII compared to controls, which was abrogated in tolerized mice (Fig. 3A, 3B). However, no modulation was found on MZ B-cells (Fig. 3C). Elevated IgE levels can lead to stabilization of CD23 (FcεRII) on B-cells, which then pick up and present allergen-IgE complexes. This is termed facilitated antigen presentation [23, 24]. Consistent with enhanced MHCII expression, spleen, bLN and lung FM B-cells from mice with an asthma-like phenotype revealed increased expression of CD23, which was downregulated in tolerized mice (Fig. 3D, 3E). Moreover, to investigate, if FM B-cells contribute to allergen-mediated T-cell proliferation, OVA-specific OT-II CD4⁺ T-cells were cultured with FM B-cells or DCs in the presence of OVA (Fig. 3F). Our data show that FM B-cells induce allergen-specific T-cell proliferation *in vitro*. As a positive control, we used DCs as APCs. As expected they produced a stronger response. The data suggest that FM B-cells contribute

to antigen presentation and allergen-specific T-cell proliferation.

Respiratory tolerance inhibits GC formation and generation of OVA-specific antibody secreting cells

Upon antigen-encounter, activated B-cells enter the GC reaction where they further differentiate into antibody-secreting plasma and memory B-cells. To investigate where OVA-specific ASC are located and if their generation is affected by tolerization, GC B-cells and OVA-specific ASC were analyzed in different lymphoid organs and OVA-specific immunoglobulins were measured in sera.

Mice with an asthma-like phenotype generated significantly more CD19⁺ peanut agglutinin (PNA)⁺ GC B-cells in spleen and bLN, whereas no modulation of GC B-cells could be observed in tolerized mice compared to controls (Fig. 4A, 4B). Consistent with this finding, elevated numbers of OVA-specific IgM-, IgA-, IgG1- and IgE-secreting cells were detectable in allergic mice, while tolerization partially inhibited the generation of OVA-specific ASC. Compared to controls, tolerized mice still had increased numbers of OVA-specific ASC (Fig. 4C-J). Overall, highest numbers of OVA-specific ASC were found within bLN. Increased numbers of OVA-specific IgG1 and IgA ASC were not only detected in the spleen, but also locally in bLN and lung as well as in BM, which were probably long living plasma cells (Fig. 4E-H). Furthermore, OVA-specific IgG1 and IgE serum levels were elevated in allergic compared to tolerized and control mice (Fig. 4K). In summary, our data demonstrate that an asthma-like phenotype causes antigen-specific antibody production, most importantly in local tissues. This can be suppressed by respiratory tolerance.

B-cells are not required for allergic airway inflammation or respiratory tolerance in an OVA model

Since our data had shown alterations of the B-cell compartment in response to allergy and respiratory tolerance, we investigated whether B-cells are necessary for the development of these processes. We measured lung function parameters resistance (R) and elastance (E), after metacholine (MCh) provocation of WT and μ MT mice. Both OVA-treated μ MT and WT mice showed a declined lung function compared to controls, demonstrated by increased R and E. These parameters were improved in tolerized mice of both genotypes (Fig. 5A-D). Furthermore, in μ MT and WT mice, OVA immunization resulted in a substantial pulmonary eosinophilia, while tolerization prevented it in both genotypes (Fig. 6A). No differences between μ MT and WT mice were detectable after induction of asthma-like or tolerant phenotype in respect to perivascular and peribronchial cell infiltrates or mucus hypersecretion and goblet cells hyperplasia analyzed by haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stained lung sections, respectively (Fig. 6B, 6C). Next, we investigated Th2 cytokine production after OVA-restimulation of cells *in vitro*. Splenocytes from allergic mice secreted significantly more IL-5 and IL-13 as compared to those from tolerized and control mice. However, there were no differences between μ MT and WT mice (Fig. 6D). Moreover, IL-5 and IL-13 secretion by bLN and lung cells of both genotypes showed comparable results (data not shown). In conclusion, our data show that μ MT mice have no differences in susceptibility to develop asthma-like or respiratory tolerant phenotype.

Discussion

In the present study we investigated, if B-cells are affected by allergic airway inflammation and respiratory tolerance and if they are necessary to develop these phenotypes. We show that B-cell subsets are influenced in spleen, bLN and lung during OVA-induced allergic airway inflammation, while respiratory tolerance diminished or completely prevented these alterations. However, development of both phenotypes does not require B-cells in our model.

In addition to alteration of transitional and naïve mature B-cell numbers, we observed increased GC B-cells, which was more pronounced in bLN compared to spleen. Similarly, Chvatchko et al. [25] demonstrated GC within the bronchus-associated lymphoid tissue and pulmonary OVA-specific ASC. When measuring OVA-specific IgM-, IgA- and particularly IgG1-ASC, we found highest numbers locally in bLN and lung. Consistent with our data, Braza et al. demonstrated HDM-mediated attraction and development of B-cells into pulmonary allergen-specific ASC [26]. In a chronic model highest numbers of cockroach-specific ASC were detected in lung, compared to LN, spleen and BM [27]. However, in contrast to our study, ASC were calculated in relation to B-cells. Luger and colleagues [28] showed in a chronic model OVA-specific IgA-, IgG1- and IgE-ASC in lung, spleen and highest level in BM. Our acute protocol showed only slightly elevated OVA-specific IgA- and IgG1-ASC level in BM than in spleen. Highest allergen-specific ASC titers were measured in bLN, which were

not investigated by Luger and coworkers. Moreover, in our model OVA-specific IgE-ASC were only detectable in spleen and bLN. An explanation for these contrasting results could be the use of different model systems. During acute phase allergen-specific ASC are mostly located in local niches of bLN and lung and also in spleen whereas in chronic phase more long-lived plasma cells are generated, that are located in the BM, where they receive survival signals [28, 29]. The majority of IgE-expressing cells are generated by sequential isotype switching [30] and even in sensitized mice OVA-specific IgE is usually very low compared to other isotypes [31]. In contrast to the previously mentioned study, we used C57BL/6J mice, which generally produce less OVA-specific IgE compared to sensitized BALB/c mice [32, 33].

B-cells in their function as APCs are involved in the pathogenesis of allergic disease [27]. In a model of cockroach-induced allergic lung disease CD19⁺ B-cells expressed higher MHCII, CD80 and CD86 levels than control B-cells. To investigate which B-cell subtypes are involved in antigen presentation we determined MHCII expression of MZ and FM B-cells. While splenic MZ B-cells showed no differences between allergic and control mice, FM B-cells significantly upregulated MCHII in spleen, but also in lung and especially bLN, which was completely abrogated in tolerized mice. Because allergen-specific IgE binds to CD23 on the B-cell surface [34], IgE/OVA complexes could be found on FM B-cells in splenic follicles [35] where they induced proliferation, activation of OVA-specific CD4⁺ T-cells and augmented antibody response in a CD23⁺B-cell dependent fashion [36], we determined CD23 expression on FM B-cells. In agreement with increased MHCII expression, FM B-cells in spleen, lung and bLN from allergic mice upregulated CD23, which was completely reversed by tolerance

induction. Moreover, FM B-cells induced OVA-specific T-cell proliferation *in vitro*. Our data suggest that FM B-cells are involved in antigen-presentation and OVA-specific T-cell proliferation and could thereby amplify the allergic response or peripheral T-cell tolerance.

Recently, regulatory functions of B-cells were investigated in more detail in models of contact hypersensitivity [37] and airway inflammation [6, 38]. Adoptive transfer of Breg cells from *S. mansoni* infected mice [39] and from hilar lymph nodes of tolerized mice [40] prevented or attenuated allergen-induced airway inflammation.

Several phenotypical characterizations of Breg cells exist. Using the characteristic phenotypic markers CD24⁺CD21⁺CD23⁺ to describe MZp B-cells, we found elevated numbers in allergic mice, but reduced MZp B-cell numbers in tolerized mice. Nevertheless, IL-10 production of total CD19⁺ B-cells from tolerized and allergic mice was similar. Since several B-cell subsets, including MZp B-cells, are described to produce IL-10, we cannot link IL-10 production exclusively with MZp B-cells and exclude that other CD19⁺ B-cell subset(s) secreted IL-10 after LPS stimulation in our *in vitro* assay.

When determining the number of MZp B-cells directly after tolerization, our data demonstrate a significant induction of MZp B-cells compared to controls. The immune regulatory control is induced directly after allergen application and the early induction of MZp B-cells supports their regulatory role in this model. Similarly, Treg cells are increased early upon tolerization compared to controls whereas repeated OVA sensitizations and challenges for several weeks result in reduced numbers of Treg cells in tolerized compared to allergic mice as previously shown in our model [17, 18]. Our

current study further demonstrated that tolerized MZp B-cells effectively induce Foxp3⁺ Treg cells suggesting that MZp B-cells could mediate regulatory function via Treg induction.

To finally analyze, if Breg cells are required for either allergic airway inflammation or tolerance induction, we compared WT to μ MT mice in our OVA model. Although distinct B-cell subsets were obviously involved in response to allergy and respiratory tolerance, B-cells seem not to be elementary for these processes. Thus, our data demonstrate comparable parameters of allergic airway inflammation including lung eosinophilia, inflammation, mucus production and *in vitro* Th2 cytokine production in WT and μ MT mice.

This situation is controversially discussed throughout the literature. Former studies demonstrate that μ MT mice can develop an OVA-induced asthma-like phenotype similar to their C57BL/6 or B10.BR counterparts based on AHR, lung eosinophilia, inflammation and mucus production, IL-4 and IL-5 secretion and T-cell responses [41-44]. In one study more pulmonary eosinophils and mucus cells are described in μ MT compared to WT mice [43]. When using a cockroach model comparing CBA/J with partially B-cell deficient Xid mice, Lundy et al. found increased cytokines, lung inflammation, eosinophilia and mucus production in Xid mice [45]. The same group demonstrated in a chronic cockroach model, now comparing C57BL/6 with μ MT, that μ MT mice exhibited decreased IL-4 and IL-5 levels and attenuated AHR, whereas no differences were found in mucus production and lung inflammation [27]. In other chronic models of OVA-induced allergic airway inflammation, using either μ MT mice on C57BL/6 background or B-cell deficient JHD mice on BALB/c background, no differences were detected between B-cell deficient and WT mice in respect to BALF, cytokines,

lung inflammation and mucus production [40, 46, 47].

In all these studies, intraperitoneal (i.p.) allergen application was followed by local inhalation challenge. Only one study used a protocol of tolerance induction similar to our study: comparing BALB/c with JHD mice, decreased T-cell responses were found in JHD mice [48]. However, no other analyses including lung function or BALF differentiation were performed. Therefore, our study shows for the first time that respiratory tolerance induction by local antigen application prior to systemic sensitization is independent of B-cells.

There are probably several regulatory mechanisms involved in maintaining and restoring immunological homeostasis when one or more factors are missing. DCs as potent APCs aggravate Th2-mediated allergic airway inflammation, but induce also Treg cells by several ways and reduce thereby inflammatory effector responses [49].

Overall, allergen and genetic background of mice seem to influence B-cell function in allergic airway inflammation. Prevention or decrease of allergic symptoms either by worm infection of mice prior to allergen sensitization or transfer of B-cells from infected or tolerized mice suggest that *in vivo* priming and/or activation of B-cells prior to allergen sensitization may be crucial for regulatory B-cell function to dampen down allergic airway inflammation.

Materials and methods

Animals

B6.129S2-Igh-6-tm1cgn/J (μ MT) mice from The Jackson Laboratory (Bar Harbor, ME, USA) and C57BL/6J WT mice from Charles River laboratories (Sulzfeld, Germany) were housed and bred under specific pathogen-free conditions in the animal facility at Hannover Medical School. All animal experiments were performed according to institutional and state guidelines and approved by the committee of animal welfare.

Induction of asthma-like phenotype and respiratory tolerance

According to protocol (Fig. 1) and as described previously [16-19] WT and μ MT mice were sensitized twice by i.p. application of 20 μ g OVA (Sigma-Aldrich, Munich, Germany; LPS content reduced <20 Endotoxin Units/mg protein) in 0.9% sodium chloride (NaCl) absorbed to aluminium and magnesium hydroxide (Imject Alum[®], Thermo Fisher Scientific, Waltham, MA, USA) followed by six i.n. OVA challenges (20 μ g OVA in 0.9% NaCl; OVA group). Control mice were treated with Alum/0.9% NaCl i.p. and 0.9% NaCl i.n. (Alum control group). To induce respiratory tolerance (TOL group) mice were pretreated twice with 500 μ g OVA in 0.9% NaCl i.n. and subsequently subjected to allergic airway inflammation protocol. Lung function measurements were performed one day after last OVA challenge, afterwards mice were sacrificed.

Lung function

Mice were anesthetized by ketaminehydrochloride (Albrecht GmbH, Aulendorf, Germany)/xylazinehydrochloride (Bayer Vital GmbH, Leverkusen, Germany), intubated and connected to flexiVent system (SCIREQ, Montreal, Canada). Mechanical ventilation was set at 180 breaths/minute with tidal volume of 10 ml/kg and positive end-expiratory pressure of 3cm H₂O. Baseline lung mechanics and AHR to nebulized MCh (5, 10 and 20 mg/ml acetyl- β -methylcholine chloride, Sigma-Aldrich, Steinheim, Germany; Aeroneb ultrasonic nebulizer, SCIREQ) were measured. Respiratory parameters were derived from the peak of dose response curves collected after application of multiple single-frequency forced-oscillations. Respiratory data are presented as mean resistance (R) in cmH₂O.s/ml, mean elastance (E) in cmH₂O/ml and mean values from area under the MCh-curve (0-20 mg/ml MCh, AUC_R, AUC_E) were calculated.

Analysis of bronchoalveolar lavage fluid (BALF)

Preparation of BALF and cell differentiation were performed as described previously [17].

Lung histology

Lung histology and quantification of pulmonary inflammation (H&E staining) and mucus production (PAS staining) were performed as described elsewhere [16].

Cell isolation

Spleens and bLN were smashed through 70 μ m mesh. Lungs were digested by 0.1 mg/ml DNaseI (Sigma-Aldrich, Munich, Germany) and 1 mg/ml collagenase diluted in RPMI+ medium (RPMI 1640 with 10% FBS, 1% penicillin/streptomycin, all Biochrom, Berlin, Germany), dissociated via GentleMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) and flushed through 30 μ m mesh. BM was yielded by centrifugation of dissected bones. Erythrocytes were lysed by Ammonium-Chloride-Potassium (ACK) buffer.

B-cells and OVA-specific OT-II T-cells were isolated by EasySep mouse B-cell isolation kit (STEMCELL Technologies, Cologne, Germany) and CD4⁺ T-cell isolation kit (Miltenyi Biotec) yielding 89-90% CD19⁺ B-cells and 91-94% CD4⁺ T-cells, respectively. DCs were enriched by Pan dendritic cell isolation kit (Miltenyi Biotec). All kits were used according to the manufacturer's instructions. FM B-cells and MZp B-cells were sorted by FACS Aria Fusion and FACS Aria IIu (BD, Heidelberg, Germany) according to the gating in figure 2A.

Determination of OVA-specific ASC by ELISPOT assay

MultiScreen_{HTS}-IP plates (Millipore, Schwalbach, Germany) were coated with 1 mg/ml OVA, blocked with RPMI++ medium (RPMI+, 1% HEPES, 100 μ M β -mercaptoethanol), cells were plated and negative controls prepared. Membranes were incubated with HRP-conjugated antibodies (IgM: 1:2000, 1B4B1; IgG1: 1:4000; SouthernBiotech, Birmingham, Alabama, USA) or Biotin-conjugated

antibodies (IgA: 1:1000, 11-44-2; IgE: 1:5000, 23G3; SouthernBiotech) followed by incubation with Streptavidin-Peroxidase Polymer (1:750; Sigma-Aldrich, Munich, Germany). Spots were developed with 3-amino-9-ethylcarbazole solution (AEC Substrate Kit for Peroxidase, Vector Laboratories, Peterborough, United Kingdom). Dried membranes were scanned by ELISPOT scanner (Eli.Scan F3200, version V2.0), analyzed by Eli.Analyse software V5.1 (all A.EL.VIS GmbH, Hannover, Germany) and relative numbers of OVA-specific ASC were calculated: spot numbers (mean of duplicates) $\times 10^6$ /plated cell numbers.

OVA-specific IgE and IgG1

Serum levels of OVA-specific IgE and IgG1 were measured by ELISA as described previously [17].

***In vitro* cytokine production and co-culture**

OVA restimulation of isolated cells and measurement of IL-5 and IL-13 in the supernatants via ELISA were described elsewhere [17]. 0.5×10^6 B-cells/well were stimulated for 3 days with 10 $\mu\text{g/ml}$ LPS (*E. coli* serotype 0111:B4, Sigma-Aldrich, Munich, Germany) in RPMI++ medium. IL-10 was measured by DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA). CFSE-labeled allergen-specific T-cells (CellTrace CFSE cell proliferation kit, Molecular Probes, Eugene, OR, USA) and FM B-cells (ratio 1:1) were incubated with 100 $\mu\text{g/ml}$ OVA for 2.5 days and proliferation was measured by FACS. For Treg

induction OVA-specific T-cells were cultured with DCs (1:10), MZp B-cells (T:B ratio 1:1) and 100µg/ml OVA for 3.5 days. Percentage of Treg cells of each condition was normalized to control condition (T-cells+DCs+OVA).

Flow cytometry and antibodies

Staining of single-cell suspensions with fluorescent antibodies, data collection and analysis were performed as described previously [17, 18]. Following antibodies were used: CD19-PerCP Cy5.5 (1D3), CD4-PerCP Cy5.5 (RM4-5; both BD, Heidelberg, Germany), CD21/CD35-PE (7E9), CD24-FITC (M1/69; both BioLegend, Fell, Germany), CD23-PE Cy7 (B3B4), MHC Class II-APC (M5/114.15.2), Foxp3-APC (FJK-16s; all eBioscience, Frankfurt, Germany), PNA-FITC (Vector Laboratories, Peterborough, United Kingdom).

Statistical analysis

For all statistical analysis GraphPad Prism software was used (GraphPad Software, La Jolla, CA, USA). Statistical significant differences (95% confidence interval) between two groups were determined by unpaired two-tailed Student's *t*-test. For comparing more than two groups two-way ANOVA (Bonferroni's multiple comparison post-test) or one-way ANOVA (Tukey's multiple comparison post-test or Kruskal-Wallis test together with Dunn's multiple comparison post-test when data showed no

Gaussian distribution) were used. Differences were considered significant at * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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AH, AMB, GH designed the research and wrote the manuscript; AH, AKB, JS, ACJ performed experiments; AH, AKB analyzed data; all authors contributed to data interpretation and commented on the manuscript.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interest.

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Figure Legends

Figure 1. Model of asthma-like and respiratory tolerance phenotypes. Mice were sensitized intraperitoneally (i.p.) with ovalbumin (OVA) absorbed to Alum and challenged intranasally (i.n.) with OVA (OVA group). Control mice (Alum group) received Alum/NaCl. Mice were tolerized with OVA i.n. and subjected to allergic airway inflammation protocol (TOL group). Mice were sacrificed after lung function measurement.

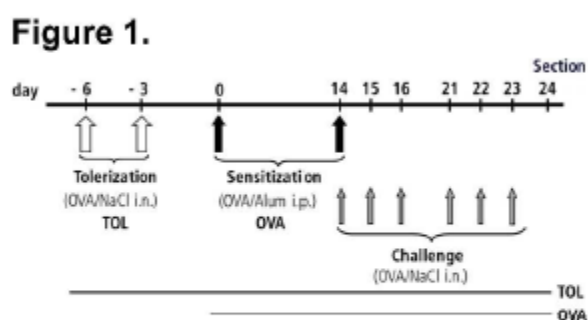


Figure 2. Allergic airway inflammation and respiratory tolerance modulate transitional and mature B-cells. (A) Flow cytometric gating strategy for $CD19^+CD21^{low}CD24^{hi}$ transitional 1 (T1) B-cells, $CD19^+CD21^{int}CD24^{hi}$ transitional 2 (T2) B-cells, $CD19^+CD21^{hi}CD24^{hi}CD23^{hi}$ marginal zone precursor (MZp) B-cells, $CD19^+CD21^{hi}CD24^{hi}CD23^{low}$ marginal zone (MZ) B-cells and $CD19^+CD21^{int}CD24^{int}$

follicular mature (FM) B-cells. (B-F) The percentages and total cell numbers of (B) T1 B-cells, (C) T2 B-cells, (D) FM B-cells, (E) MZ B-cells, (F) MZp B-cells in the indicated organs among indicated groups are shown (n=15-17/group, pooled from 3 independent experiments). Statistical significances were determined by one-way ANOVA. Tukey's test - T1: percentages bronchial lymph nodes (bLN, B top); T2: percentages (C, top); FM: spleen (D), bLN (D, left); MZ: percentages (E, left); MZp (F). Kruskal-Wallis/Dunn's test - T1: spleen (B), total numbers bLN (B, bottom); T2: total numbers (C, bottom); FM: lung (D, left); MZ: total numbers (E, right). (G) Splenic B-cells were stimulated with LPS and IL-10 was analyzed by ELISA (n=9-12/group from 1 experiment representative of 3 independent experiments, one-way ANOVA/Tukey's test). (H) Splenic MZp B-cells from control (ctrl.) and tolerized (tol.) mice were analyzed on day 0 by flow cytometry. Percentages (left) and total cell numbers (right) are presented (n=5-6/group from 1 experiment representative of 2 independent experiments, unpaired two-tailed Student's t-test). (I-J) MZp B-cells from control and tolerized mice were isolated on day 0 and cultured with allergen-specific CD4⁺ T-cells together with dendritic cells (DCs) and OVA. CD4⁺Foxp3⁺ regulatory T (Treg) cells for each condition are shown as (I) representative flow cytometry plot and (J) relative increase of control. Data shown are from 2 independent experiments (n=2, cells pooled from 4-6 mice/sample). (B-H, J) Results are expressed as mean $\pm$ SEM. * p<0.05, ** p<0.01, *** p<0.001. Alum= negative control, OVA= ovalbumin-immunized mice, TOL= tolerized mice.

Figure 2.

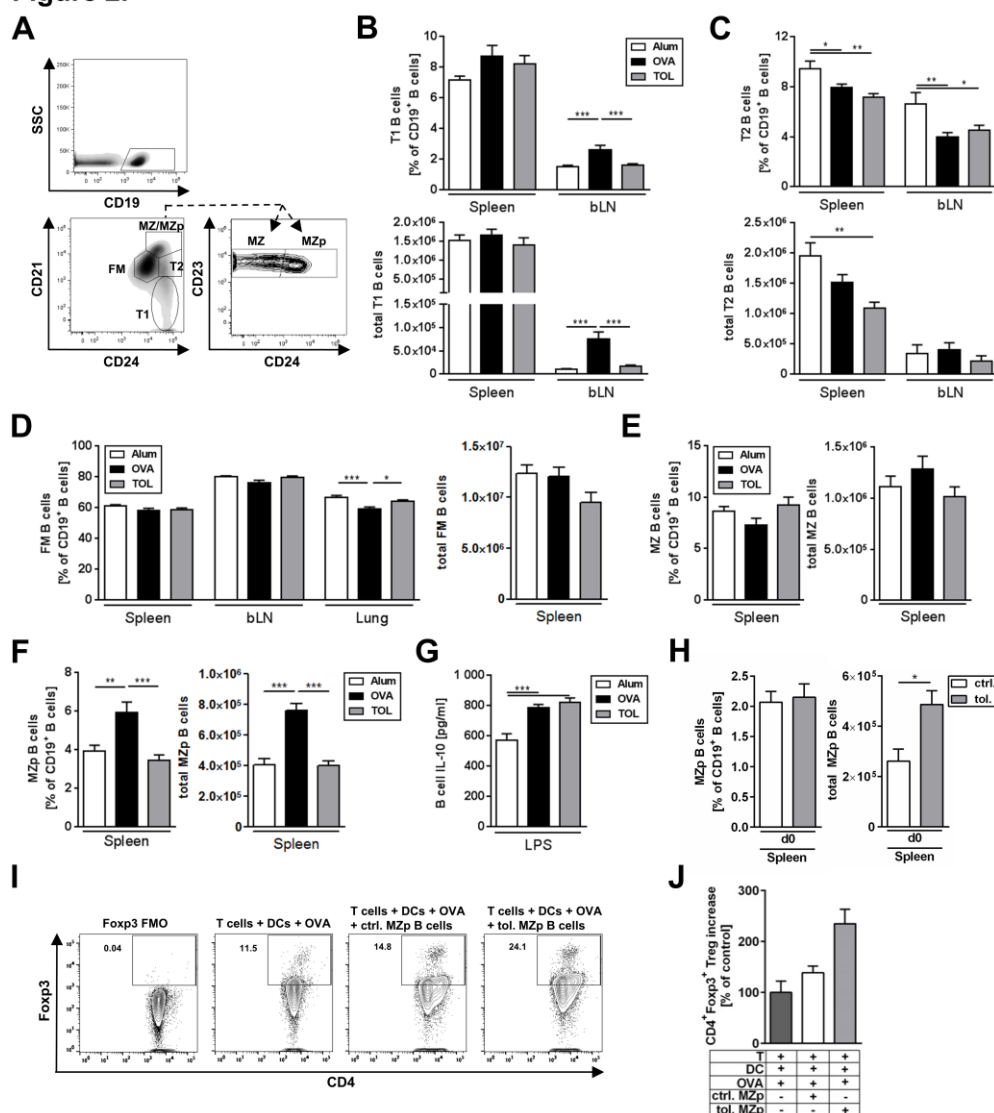


Figure 3. FM B-cells are involved in antigen presentation. (A-E) WT cells were stained for analysis of MHCII and CD23 expression, presented as mean fluorescence intensity (MFI). Flow cytometric gating of FM and MZ B-cells is as shown in figure 2A. (A-B) MHCII expression on FM B-cells is presented as (A) representative histogram and (B) bar graph (n=6/group). (C) Expression of MHCII on MZ B-cells is shown as characteristic flow cytometric plot (left) and bar graph (right, n=6/group). (D-E) CD23 expression on FM B-cells is presented as (D) representative flow cytometry plots and (E) bar graph (n=6/group). (B, C, E) Data are shown as mean + SEM from 1 experiment representative of 3 independent experiments. Statistical significances were determined by one-way ANOVA. Tukey's test - MHCII on FM (lung); on MZ (spleen); CD23 on FM (spleen, bLN). Kruskal-Wallis/Dunn's test - MHCII on FM (spleen, bronchial lymph nodes, bLN); CD23 on FM (lung). (F) FM B-cells were cultured with allergen-specific CD4⁺ T-cells in OVA presence and absence. Allergen-mediated proliferation was measured by CFSE. Representative flow cytometry plot (left), relative and total proliferation (right) are shown. Results presented as mean + SEM (n=3, cells pooled from 2 mice/sample) from 1 experiment representative of 2 independent experiments (one-way ANOVA/Tukey's test). * p<0.05, ** p<0.01, *** p<0.001. FMO= Fluorescence minus one control, Alum= negative control, DC= dendritic cell, OVA= ovalbumin-immunized mice, TOL= tolerized mice.

Figure 3.

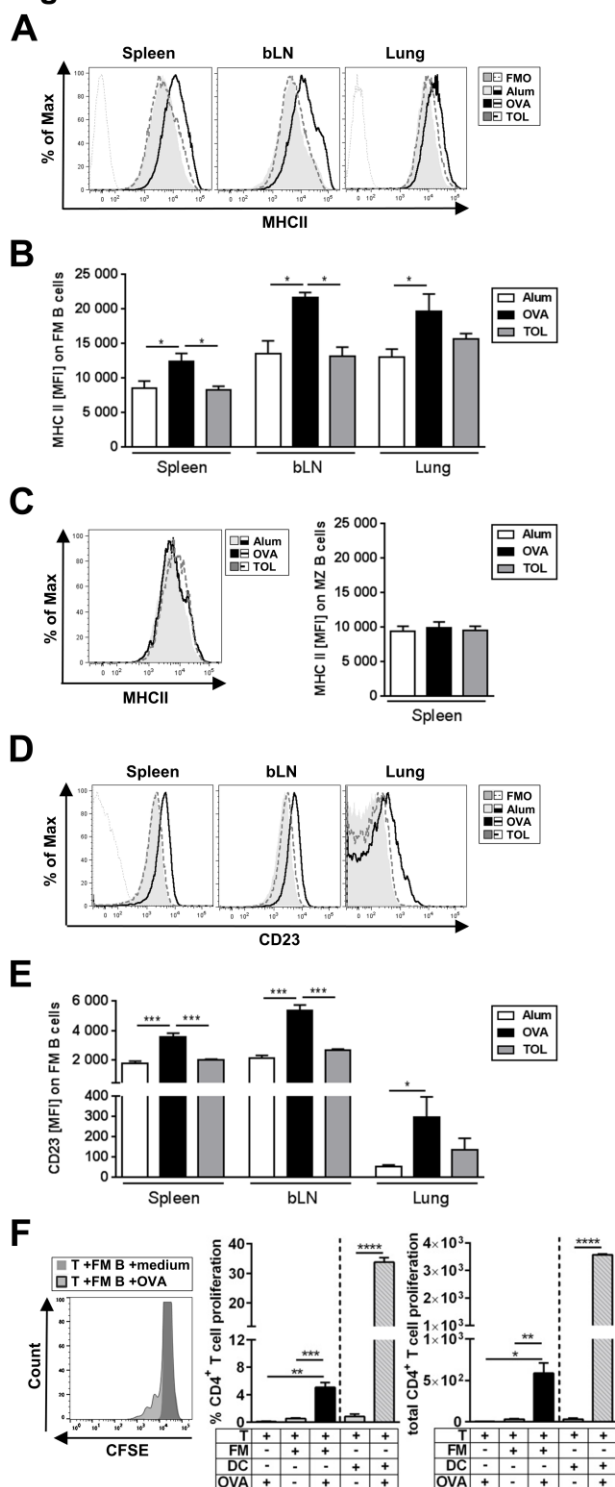


Figure 4. Respiratory tolerance attenuates raised numbers of germinal center (GC) B-cells and allergen-specific antibody secreting cells (ASC). (A-B) CD19⁺PNA⁺ GC B-cells of WT mice were analyzed by flow cytometry. (A) Characteristic flow cytometry plots, (B) percentages (top, n=15-16/group) and total cell numbers (bottom, n=11-16/group) pooled from 3 independent experiments are presented. Statistical significances were determined by one-way ANOVA/Kruskal-Wallis/Dunn's test. (C-J) OVA-specific ASC were examined by ELISPOT assay. Representative pictures (top) and bar graphs (bottom) are shown from (C, D) IgM, (E, F) IgA, (G, H) IgG1, (I, J) IgE. (D, F, H, J) Data presented were pooled from 2 (BM, spleen: n=13-16/group), 3 (lung: n=13-16/group) and 4 (bronchial lymph nodes, bLN: n=7-10/group) independent experiments. Analysis was performed by one-way ANOVA. Tukey's test - IgM (bLN); IgG1 (spleen, bLN, lung). Kruskal-Wallis/Dunn's test - IgM (BM, spleen, lung); IgA (all organs); IgG1 (BM); IgE (all organs). (K) OVA-specific IgG1 and IgE serum levels were measured by ELISA and calculated as titer (IgG1, n=9-15/group pooled from 3 independent experiments, one-way ANOVA/Kruskal-Wallis/Dunn's test) or concentration (IgE, n=3-6/group from 1 experiment representative of 2 experiments, one-way ANOVA/Tukey's test). (B, D, F, H, J, K) Data are expressed as mean $\pm$ SEM. * p<0.05, ** p<0.01, *** p<0.001. Alum= negative control, OVA= ovalbumin-immunized mice, TOL= mice tolerized.

Figure 4.

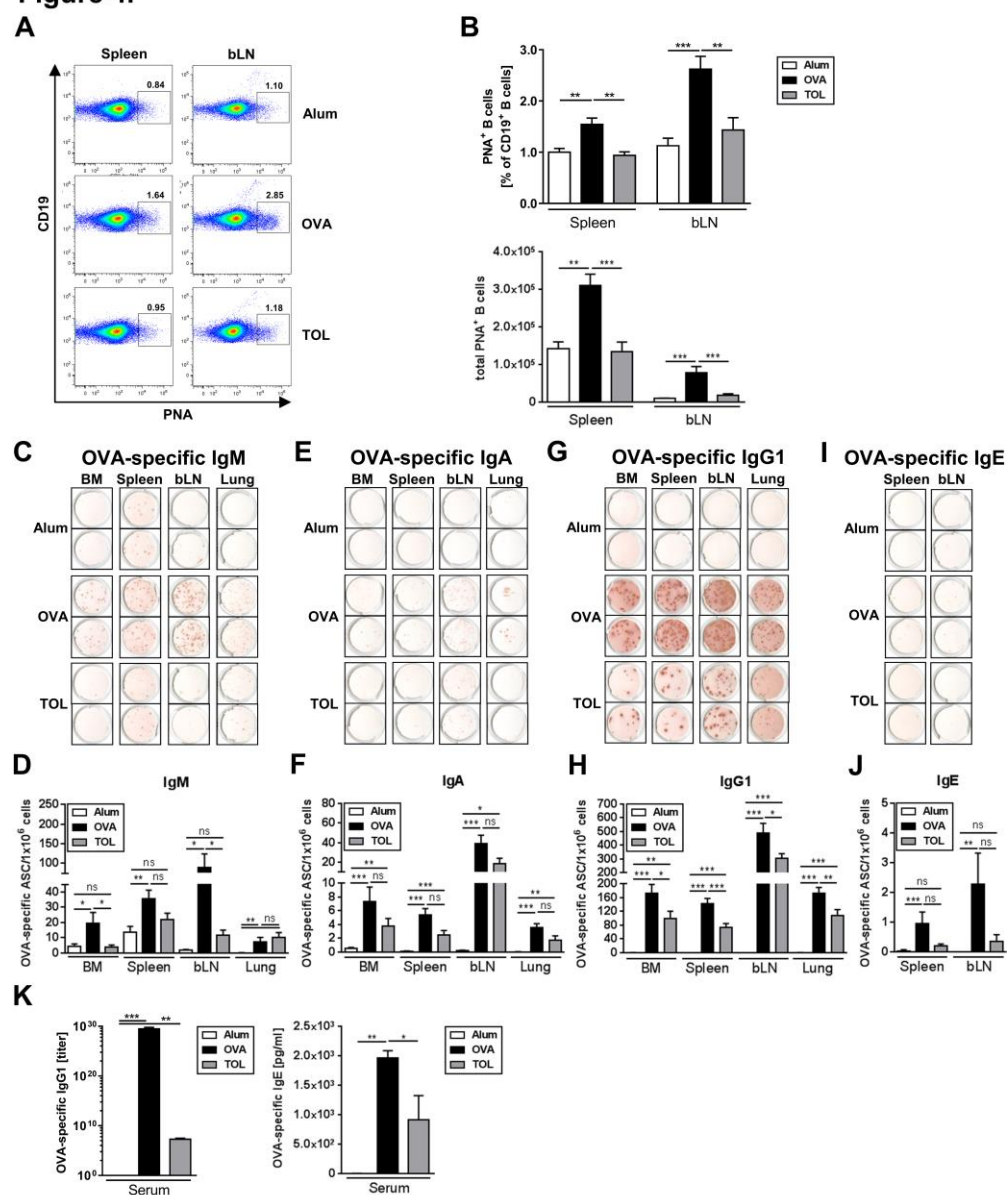


Figure 5. Tolerance induction improves airway hyperreactivity (AHR) of allergic WT and B-cell deficient (μ MT) mice. AHR of allergic, tolerized and control μ MT and WT mice was analyzed by measurement of the lung function parameters resistance (R) and elastance (E) after methacholine (MCh) provocation. R, E and area under the MCh response curves of R and E of (A, C) WT (n=4-5/group) and (B, D) μ M mice (n=7-10/group) are pooled from 2 experiments and shown as mean + SEM. Statistical significance between groups in R and E measurements were determined by two-way ANOVA/Bonferroni's test; in AUC_R and AUC_E by one-way ANOVA/Tukey's test. # Alum vs OVA; * OVA vs TOL. #/* $p < 0.05$, ##/** $p < 0.01$, ###/*** $p < 0.001$. Alum= negative control, OVA= ovalbumin-immunized mice, TOL= tolerized mice.

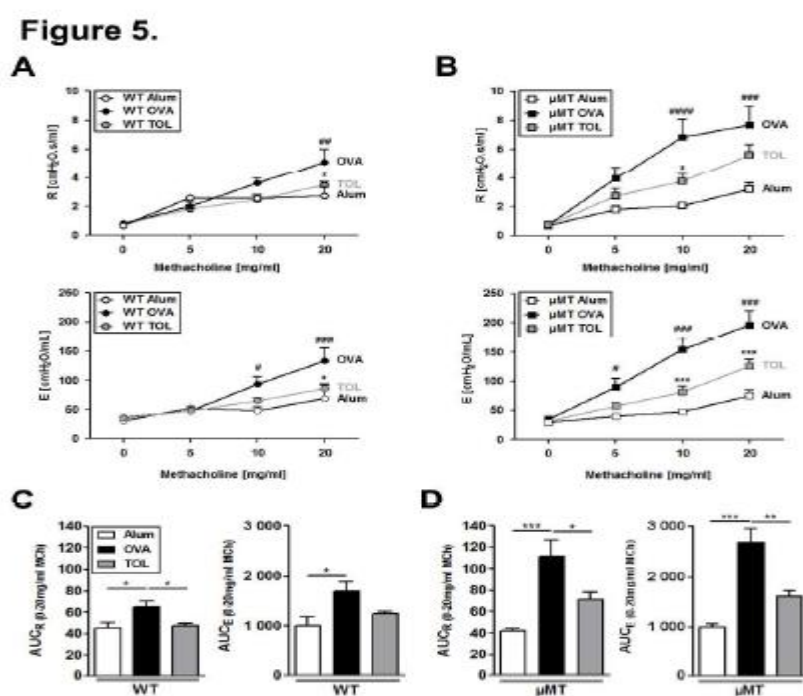
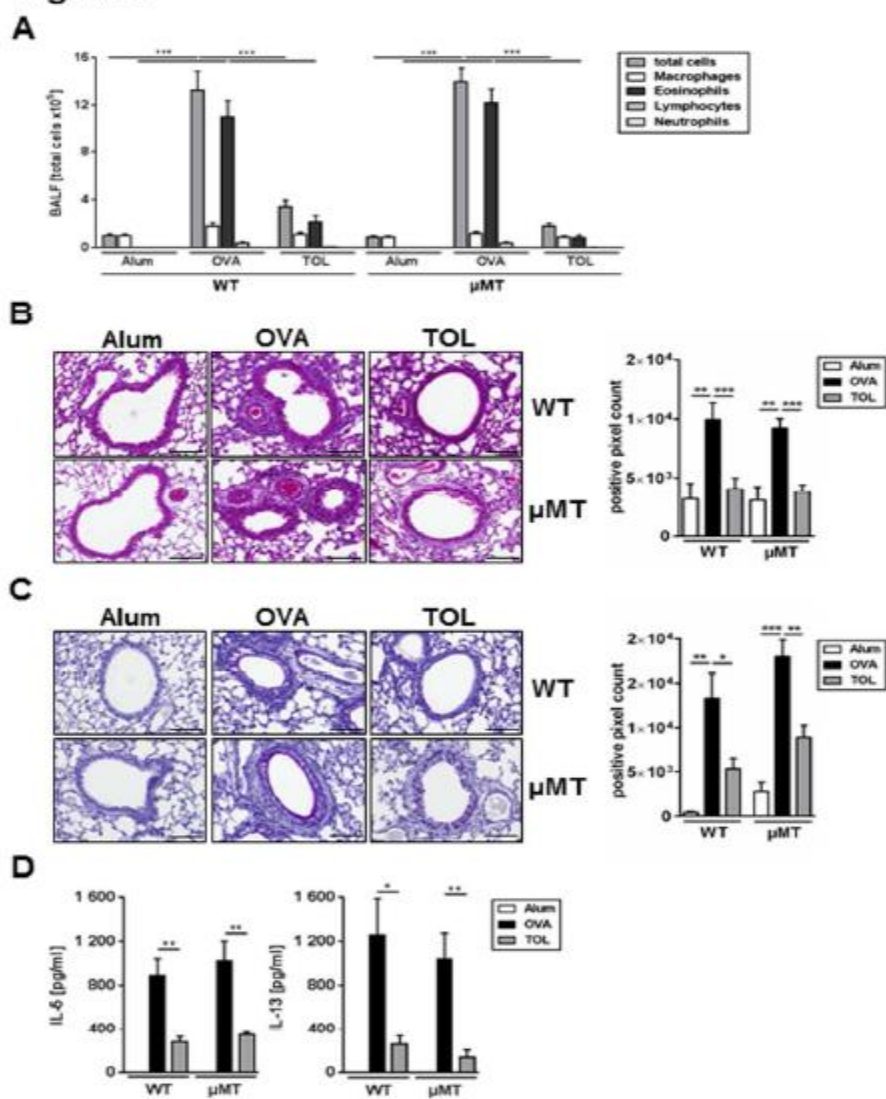


Figure 6. μ MT mice develop asthma-like and respiratory tolerant phenotypes like WT mice. (A)

Bronchoalveolar lavage was performed in allergic, respiratory tolerant and control μ MT and WT mice. BALF cells were differentiated morphometrically after May-Grünwald/Giemsa staining. Results shown as mean + SEM are pooled from 3 independent experiments (n=11-15/group, two-way ANOVA/Bonferroni's test). (B-C) Lung sections were stained with (B) H&E and (C) PAS to analyze pulmonary inflammation and mucus production respectively. HistoClick Software was used to quantify positive pixel staining (right, n=5-13/group, one-way ANOVA/Tukey's test). Magnification=x200, scale bar=100 μ m. (D) Splenic cells were restimulated with OVA and IL-5 and IL-13 production were measured by ELISA (n=8-9/group, one-way ANOVA/Kruskal-Wallis/Dunn's test). (B-D) Data expressed as mean $\pm$ SEM are pooled from 2 independent experiments. * p<0.05, ** p<0.01, *** p<0.001. Alum= negative control, OVA= ovalbumin-immunized mice, TOL= tolerized mice.

Figure 6.



B-cell subsets are modulated during allergic airway inflammation but are not required for the development of respiratory tolerance in a murine model

Anika Habener, Ann-Kathrin Behrendt, Jelena Skuljec, Adan Chari Jirno, Almut Meyer-Bahlburg and Gesine Hansen

Corresponding author: Gesine Hansen, Department of Paediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Hannover, Germany

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 25 July 2016

Dear Prof. Hansen, Prof. Meyer-Bahlburg and Mrs. Habener,

Please accept my sincerest apologies for the prolonged delay in processing the peer review of your manuscript ID ej.201646518 entitled "B cells are modulated by asthma but are not required for the development of respiratory tolerance in a murine experimental asthma model" which you submitted to the European Journal of Immunology. There was a substantial delay in selecting referees due to Executive Editor (un)availability, and thereafter a severe delay in one of the referee reports. We have since consulted with our Editor-in-Chief; the comments of the referees are included at the bottom of this letter. You should know that this is not the usual turnaround time for peer review at the Journal and we will strive to do better at the next round of revision!

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. You will see that both referees appreciate your study and that referee 1 feels that some more functional characterization is necessary. The Editor-in-Chief agrees with this assessment.

Peer review correspondence

Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Hans-Martin Jack and Prof. Francesco Annunziato

Dr. Karen Chu
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Reviewer: 1

Comments to the Author

The manuscript "B cells are modulated by asthma but are not required for the development of respiratory tolerance in a murine experimental asthma model" by Anika Habener et al and supervised by Almut Meyer-Bahlburg and Gesine Hansen is potentially interesting as the role of B cells in asthma and tolerance induction is of pathological importance.

The authors observed many changes in the percentage and number of B cell subsets after OVA immunization and that follicular mature B cells from lung, bronchial lymph nodes (bLN) and spleen exhibited increased MHCII and CD23 expression. The numbers of germinal center B cells were elevated and associated with an increased production of allergen-specific immunoglobulins especially in bLN. They inform that the numbers of splenic regulatory marginal zone precursor B cells were increased, in both group of mice (with asthma and tolerant).

They observed that in contrast to mice that develop asthma, mice with respiratory tolerance clearly attenuated all B cell changes.

Using wildtype (WT) and B cell deficient (μ MT) mice the authors investigated if B cells are necessary to develop asthma and tolerance. However, μ MT mice developed asthma-like and tolerized phenotypes similar to WT. The results clearly indicate that B cells are not required for the development of asthma and also for the development of respiratory tolerance.

Instead of the relevance of the results, I have the following comments:

1. Contrary to the expected role of regulatory B cells, MZp subset was increased in bronchial lymph nodes of asthmatic mice, whereas it was reduced by development of respiratory tolerance. The question is, have MZp B cells regulatory function in the asthma model? or you might think that this population has a pathogenic role?

The authors inform that there are changes in "Regulatory B cells" however they do not test the regulatory function of B cells in tolerant mice and/or in mice with asthma. They assume the regulatory function only based in the phenotype of the population and IL-10 production.

In my experience, IL-10 producing B cells are detected in many experimental models (infections and autoimmunity) but their role can be influenced by other cytokine-producing cells that counteract the regulatory function.

Interestingly the author indicate that "Consistent with an increase of regulatory MZp, splenic B cells isolated from allergic mice secreted higher IL-10 amounts after in vitro stimulation with lipopolysaccharide (LPS) compared to B cells isolated from control mice. However, the decrease of MZp B cells in tolerized mice is not accompanied by diminished IL-10 production by B cells. Based on this result I think it is unlikely that MZp population contributes with IL-10 production.

I think that the authors should functionally characterize this population considering this population decrease in tolerant mice. To talk about regulatory B cells the authors must test regulatory function. In addition the authors should discuss why tolerant mice have less regulatory B cells, if they consider that MZp B cells have regulatory function.

2. In addition the authors mention that "In addition to immunoglobulin and cytokine production, B cells can act as professional APC. To test their ability for antigen presentation, expression of MHCII molecules on FM and MZ B cells was examined.

Peer review correspondence

Again, the antigen-presenting capacity should be evaluated in an in vitro assay. Many molecules are involved in antigen-presentation, not only MHCII and CD23. There are many molecules, including cytokines, involved in antigen presentation (with positive function or with inhibitory function) that were not evaluated.

3. Finally, in μ MT and WT mice, OVA immunization resulted in a substantial eosinophilic influx into the lung, while tolerization with OVA prevented eosinophilia in both genotypes. This result suggests that there is a population of cells that promotes the influx of eosinophils that can be induced by B lymphocytes in WT, but in μ MT mice other Ag-presenting cells have this function.

Considering that the changes in B cells observed in mice with asthma are reverted in tolerant mice, the authors have a mice model to study changes in other non-B cell populations.

Reviewer: 2

Comments to the Author

The authors extend their previous work by demonstrating that (i) B cell subsets differ between mice with and without allergic airway inflammation, (ii) tolerance induction by inhalatory challenge with allergen prior to sensitization reduces several of the effects, and (iii) symptoms of allergic sensitization also occur in the absence of B cells.

While the latter observation essentially reconfirms classical experiments from the 1990ies, the first two points add to the understanding of allergic sensitization and (experimental) tolerance induction in the mouse. In particular it is of importance that the authors compare the various lymphatic compartments such as lung, bronchial lymph nodes, and spleen. The authors use appropriate controls by studying mice that were mock sensitized using Alum/NaClO₂ 9%. Since various mouse strains, lymphatic compartments and cell subpopulations were studied, this work summarizes a set of experiments of considerable size.

In summary, these experiments confirm that B cells subsets are affected by allergic sensitization and tolerance induction, but that their role in asthma remains unclear because in this experimental model the clinical effects do not depend on the B cell subsets.

Minor Comments

1. Throughout the manuscript it would be preferable to avoid the term “allergic asthma” in mice (e.g. abstract, line 14, page 6 line 30, page 10 line 7 and Figure legend 2, line 21. I would recommend the terms “asthma-like phenotype” or “allergic airway inflammation”. The title should be revised: “B cells are modulated by asthma” should be changed to (for example): “B cell subsets are modulated during allergic airway inflammation but ...”.

2. Page 9 line 41: “...to develop of...” please correct grammar.

3. Page 10 line 51: “An explanation for this, in part, contrasting results could” please correct grammar.

4. Figure 3A, C, and D: formatting error in the legends.

First revision – authors' response – 1 November 2016

Reviewer: 1

Instead of the relevance of the results, I have the following comments:

1. Contrary to the expected role of regulatory B cells, MZp subset was increased in bronchial lymph nodes of asthmatic mice, whereas it was reduced by development of respiratory tolerance. The question is, have MZp B cells regulatory function in the asthma model? or you might think that this population has a pathogenic role?

The authors inform that there are changes in "Regulatory B cells" however they do not test the regulatory function of B cells in tolerant mice and/or in mice with asthma. They assume the regulatory function only based in the phenotype of the population and IL-10 production.

In my experience, IL-10 producing B cells are detected in many experimental models (infections and autoimmunity) but their role can be influenced by other cytokine-producing cells that counteract the regulatory function.

Interestingly the author indicate that "Consistent with an increase of regulatory MZp, splenic B cells isolated from allergic mice secreted higher IL-10 amounts after in vitro stimulation with lipopolysaccharide (LPS) compared to B cells isolated from control mice. However, the decrease of MZp B cells in tolerized mice is not accompanied by diminished IL-10 production by B cells. Based on this result I think it is unlikely that MZp population contributes with IL-10 production. I think that the authors should functionally characterize this population considering this population decrease in tolerant mice.

To talk about regulatory B cells the authors must test regulatory function. In addition the authors should discuss why tolerant mice have less regulatory B cells, if they consider that MZp B cells have regulatory function.

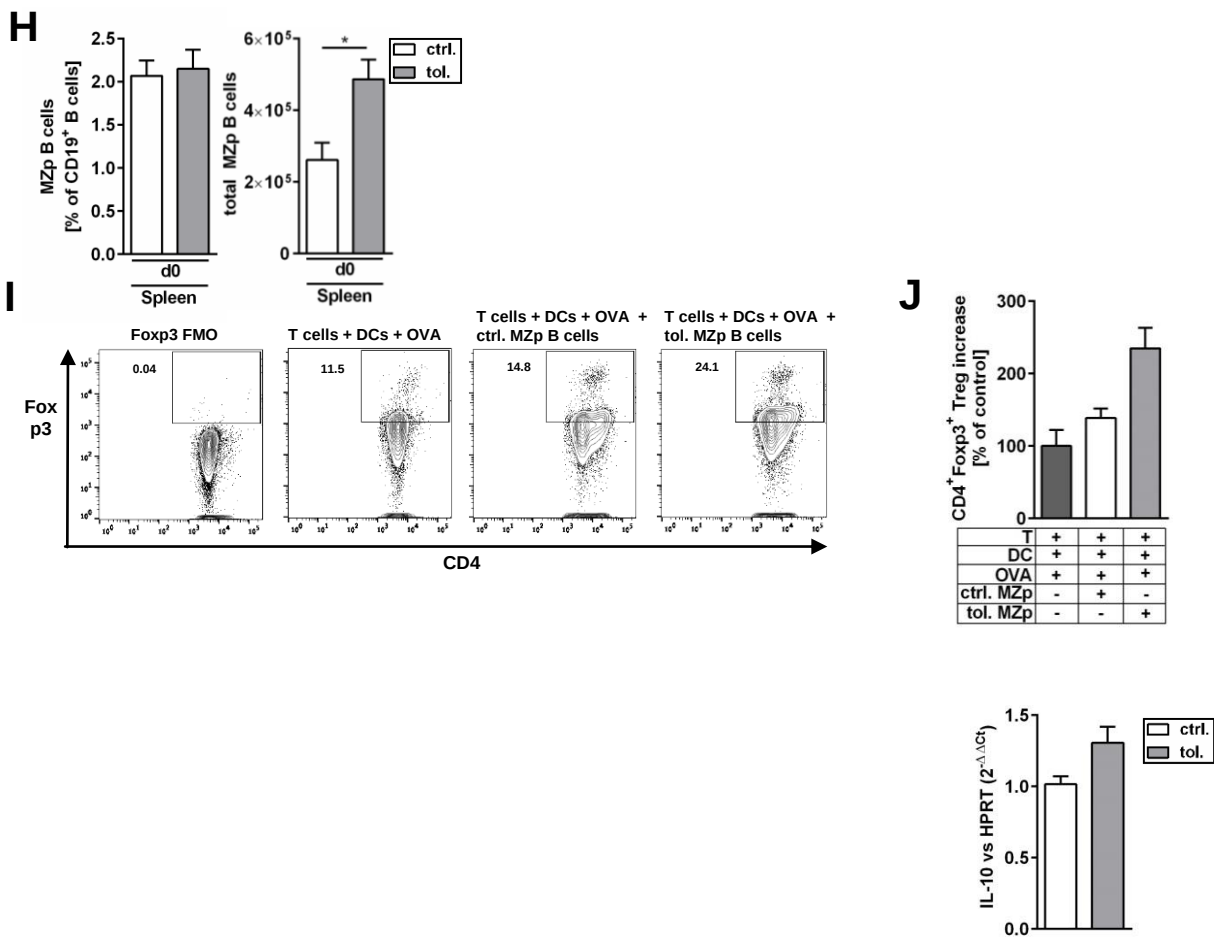
Response:

We thank the reviewer for these helpful comments. We tried to respond to these suggestions with multiple points:

a) Regulatory function of MZp B cells:

In order to functionally characterize MZp B cells, we investigated the regulatory potential of MZp B cells early in the experimental model after in vivo tolerance induction. Our data show that MZp B cells from tolerized but not control mice can effectively induce Tregs. We described the new findings on pages 3, 6, 11 and Fig. 2H-J:

... “We next directly tested the capability of MZp B cells to induce Tregs which is one mechanism of Bregs to exert their regulatory function [23, 24]. For these experiments, MZp B cells were isolated from mice directly after tolerization (day 0, Fig. 1). Notably, at this early time point, absolute numbers of MZp B cells are increased in tolerized compared to control mice, suggesting that they are directly induced upon tolerization (Fig. 2H). When MZp B cells were cultured with OVA-specific CD4⁺ T cells in the presence of dendritic cells (DCs) and OVA, MZp B cells from tolerized mice induced more Foxp3⁺ Tregs compared to control cells in vitro (Fig 2I, 2J).”...



In addition, in the course of this review we further characterized MZp B cells after tolerance induction in vivo. Preliminary data of sorted MZp B cells show that tolerized (tol.) cells expressed more IL-10 mRNA compared to control (ctrl.) cells, suggesting that MZp B cells could mediate regulatory function via Treg induction and possibly IL-10.

Additionally, we discussed MZp B cell numbers and IL-10 production by total CD19⁺ B cells on page 6, 10 and 11.

b) MZp B cell numbers:

...“Using the characteristic phenotypic markers CD24⁺CD21⁺CD23⁺ to describe MZp B cells, we found elevated numbers in allergic mice, but reduced MZp numbers in tolerized mice. However, when determining the number of MZp B cells directly after tolerization, our data demonstrate a significant induction of MZp B cells compared to controls. The immune regulatory control is induced directly after allergen application and the early induction of MZp B cells supports their regulatory role in this model. Similarly, Tregs are increased early upon tolerization compared to controls whereas repeated OVA sensitizations and challenges for several weeks result in reduced numbers of Tregs in tolerized compared to allergic mice as previously shown in our model [18, 19].

c) IL-10 production by total CD19⁺ B cells:

... Despite reduced numbers of MZp B cells in tolerized mice, total CD19⁺ B cells from allergic and tolerized mice generated similar amounts of IL-10, which was significantly higher compared to B cells from control mice (Fig. 2G).” ...

... “several B cell subsets, including MZp B cells, are described to produce IL-10. Therefore, we cannot link IL-10 production exclusively with MZp B cells and exclude that other CD19⁺ B cell subset(s) secreted IL-10 after LPS stimulation in our in vitro assay.” ...

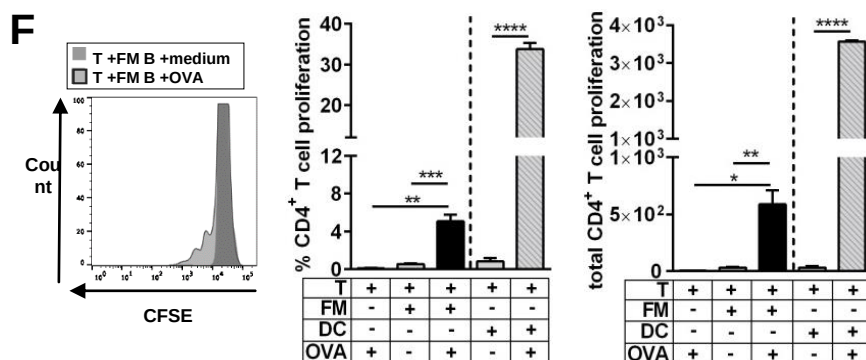
In addition, it could be possible, that allergic B cells are restricted in their ability to produce IL-10 compared to tolerized B cells.

2. In addition the authors mention that “In addition to immunoglobulin and cytokine production, B cells can act as professional APC. To test their ability for antigen presentation, expression of MHCII molecules on FM and MZ B cells was examined.

Again, the antigen-presenting capacity should be evaluated in an in vitro assay.

Response:

In order to test the antigen-presenting capacity of FM B cells, we cultured OVA-specific OT-II T cells with FM B cells (ratio 1:1) in presence of OVA and measured proliferation of CD4⁺ T cells. FM B cells are able to induce allergen-specific T cell proliferation in vitro. New results are described in pages 3, 7 and 10 and shown in Figure 3F.



...“Moreover, to investigate, if FM B cells contribute to allergen-mediated T cell proliferation, OVA-specific OT-II CD4⁺ T cells were cultured with FM B cells or DCs in the presence of OVA (Fig. 3F). Our data show that FM B cells induce allergen-specific T cell proliferation in vitro. As a positive control, we used DCs as APCs. As expected they produced a stronger response. The data suggest that FM B cells contribute to antigen presentation and allergen-specific T cell proliferation.”...

3. Finally, in μ MT and WT mice, OVA immunization resulted in a substantial eosinophilic influx into the lung, while tolerization with OVA prevented eosinophilia in both genotypes. This result suggests that there is a population of cells that promotes the influx of eosinophils that can be induced by B lymphocytes in WT, but in μ MT mice other Ag-presenting cells have this function.

Considering that the changes in B cells observed in mice with asthma are reverted in tolerant mice, the authors have a mice model to study changes in other non-B cell populations.

Response:

We agree with the reviewer that this could be a useful model to study non-B cell Ag-presenting cells and thank him for this inspiring comment.

Our current study clearly focusses on B cells and thus, investigation of other cell types was beyond the scope of this work. With regard to further studies, we discussed dendritic cells as interesting Ag-presenting cells which contribute to Th2-mediated allergic airway inflammation and tolerance on page 12. In addition, previous work from our lab pointed to the important role of costimulatory networks in allergic airway inflammation and respiratory tolerance, including CD30/CD153 pathway (Polte et al., J Allergy Clin Immunol, 2006) and ICOS (Busse et al., J Immunol, 2012).

...“There are probably several regulatory mechanisms involved in maintaining and restoring immunological homeostasis when one or more factors are missing. DCs as potent APCs aggravate Th2-mediated allergic airway inflammation, but induce also Tregs by several ways and reduce thereby

inflammatory effector responses [50].”...

Reviewer: 2

Minor Comments

1. Throughout the manuscript it would be preferable to avoid the term “allergic asthma” in mice (e.g. abstract, line 14, page 6 line 30, page 10 line 7 and Figure legend 2, line 21. I would recommend the terms “asthma-like phenotype” or “allergic airway inflammation”. The title should be revised: “B cells are modulated by asthma” should be changed to (for example): “B cell subsets are modulated during allergic airway inflammation but ... ”.

Response:

We replaced the term “allergic asthma” in mice in the whole text.

Original title: “B cells are modulated by asthma but are not required for the development of respiratory tolerance in a murine experimental asthma model”,

revised title: “B cell subsets are modulated during allergic airway inflammation but are not required for the development of respiratory tolerance in a murine model”.

2. Page 9 line 41: “...to develop of...” please correct grammar.

Response:

We corrected the grammar: “...to develop asthma-like or ...”.

3. Page 10 line 51: “An explanation for this, in part, contrasting results could” please correct grammar.

Response:

The reviewer is right. We corrected the grammar: “An explanation for these contrasting results could be...”.

4. Figure 3A, C, and D: formatting error in the legends.

Response:

We apologize for this mistake. We rearranged Figure 3 legend.

Figure 3. Respiratory tolerance prevents allergy-induced expression of MHCII and CD23 on FM B cells. WT cells were stained for analysis of MHCII and CD23 expression, presented as mean

fluorescence intensity (MFI). FACS gating of FM and MZ B cells is shown in figure 2A. (A-B) MHCII expression on FM B cells is presented as representative FACS plots (A) and bar graph (B). (C) Expression of MHCII on MZ B cells is shown as characteristic FACS plot (left) and bar graph (right). (D-E) CD23 expression on FM B cells is presented as MFI by representative FACS plots (D) and bar graph (E).

Second Editorial Decision – 24 November 2016

Dear Prof. Hansen,

It is a pleasure to provisionally accept your manuscript entitled "B cell subsets are modulated during allergic airway inflammation but are not required for the development of respiratory tolerance in a murine model" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Hans-Martin Jäck

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