



# CD27 costimulation is not critical for the development of asthma and respiratory tolerance in a murine model

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## ABSTRACT

CD27 is a costimulatory molecule of the TNFR family strongly expressed on activated CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. Binding with its ligand CD70, present on lymphocytes and DCs, leads to enhanced T cell activation and proliferation. Several other costimulatory molecules of the TNFR family like CD30, CD134 (OX40) or CD137 (4-1BB) have been shown to be critically involved in the development of asthma and/or respiratory tolerance. However, the role of CD27/CD70 signalling in these disease models has not been studied intensively. The aim of this study was to directly investigate the role of CD27 for the development of asthma and respiratory tolerance by comparative analysis of wild type (WT) and CD27<sup>-/-</sup> mice in the corresponding murine models. Ovalbumin (OVA)-sensitized and challenged CD27<sup>-/-</sup> mice developed comparably increased airway hyperreactivity (AHR), eosinophilic airway inflammation, mucus hypersecretion and elevated OVA-specific serum IgE levels in response to OVA sensitization as WT mice. In addition, Th2 cytokine production in spleen cell culture supernatants and proliferation of splenocytes after *in vitro* OVA restimulation was equally enhanced when derived from WT and CD27<sup>-/-</sup> mice. Furthermore, the absence of CD27 had no decisive impact on tolerance induction, so that WT and CD27<sup>-/-</sup> mice were comparably protected from asthma development by mucosal antigen application before sensitization. Our results suggest that CD27 costimulation is dispensable for a Th2 cell mediated allergic asthma response and respiratory tolerance induction in murine models.

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## 1. Introduction

The majority of the human population is characterized by unresponsiveness against common respiratory antigens and is therefore protected against the development of allergic diseases. This tolerance is achieved after repeated mucosal contact to airborne allergens by inhalation and is thought to be mediated by different mechanisms including T cell clonal deletion, anergy and suppression [1]. However, an increasing part of the western civilization suffers from allergic diseases like atopic dermatitis, hay fever, food allergies and allergic asthma [2,3]. Allergic asthma is a chronic disorder characterized by episodic reversible airway obstruction, bronchial hyperresponsiveness and eosinophilic pul-

monary inflammation. The inflammatory process is mainly driven by the overproduction of cytokines, e.g. IL-4, IL-5 and IL-13 of allergen-specific Th2 cells.

The antigen-specific activation of a naïve T cell is known to be the initial step of both Th2-polarized immune reactions leading to disease pathology, as well as tolerogenic responses that protect against allergic asthma [4]. For optimal T cell activation not only the recognition of the antigen presented on MHC class II complex on dendritic cells but also the concurrent signalling through costimulatory molecules is compulsory [5,6]. One of these molecules is CD27, a costimulatory molecule of the TNFR superfamily [7,8], which includes the molecules CD30, CD40, CD134 (OX40), HVEM and CD137 (4-1BB). CD27 is a type I transmembrane protein expressed on T and B lymphocytes, as well as natural killer (NK) and hematopoietic progenitor cells [9,10]. The ligand for CD27 is CD70 [11], a member of the TNF family, which is expressed on activated B cells and T cells, particularly on activated CD4<sup>+</sup> CD45RO<sup>+</sup> T cells [12]. While the involvement of CD27/CD70 signalling in the differentiation of B cells into plasma cells has been described [13,14], the role of CD27 as costimulatory molecule in IgE-mediated allergic diseases is poorly defined. Nagumo et al. reported that CD27/CD70

**Abbreviations:** AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; bLNs, bronchial lymph nodes; DC, dendritic cell; EC, experimental allergic conjunctivitis; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; Penh, enhanced pause; TNFR, tumour necrosis factor receptor; WT, wild type.

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costimulation promotes B cell proliferation and differentiation into plasma cells leading to an increase in IgE secretion [14]. However, recently it has been shown that CD27 and CD70 are not critical for the development of experimental allergic conjunctivitis (EC) in a murine model [15].

In murine models of allergic asthma several costimulatory molecules of the TNFR family like CD30 [16,17], OX40 [18,19] and CD137 [20–22] are critically involved in the development of this Th2 cell mediated disease [23]. However, to our knowledge CD27 has not been investigated in asthma models yet. The aim of the present study was to directly investigate the role of CD27/CD70 costimulation in the development of Th2-mediated allergic asthma and respiratory tolerance by comparing CD27<sup>-/-</sup> mice and WT mice in corresponding murine models. Our data show, that in contrast to other members of the TNFR receptor family like CD30 [17], OX40 [19] and CD137 [20], CD27 signalling is dispensable for the development of asthma in the murine model. CD27<sup>-/-</sup> mice developed the complete asthma phenotype after sensitization and pulmonary challenge with a model allergen comparable to WT mice. Moreover, our analysis in a model of respiratory tolerance, as contrary immunologic response, showed that CD27 deficiency does not impair the induction of respiratory tolerance since CD27<sup>-/-</sup> mice were equally protected from the development of asthma after mucosal antigen application before sensitization as WT mice. In summary our data suggest that CD27 in contrast to other members of the TNFR family is not crucially involved in the development of asthma and respiratory tolerance in murine models.

## 2. Materials and methods

### 2.1. Mice

WT C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and CD27<sup>-/-</sup> mice (B6.129P2-CD27<sup>tm1Jbo</sup>) were obtained from the Animal Facility of Bern University Hospital (Prof. Dr. Adrian Ochsenbein, Inselspital Bern, Switzerland; originally generated by Prof. Dr. Jannie Borst from the Netherlands Cancer Institute, Amsterdam, Netherlands). Due to an infection with MHV, CD27 mice were hygienically rederived by cesarean section and fostered by germ free Swiss Webster mice. One male pup could be weaned and was backcrossed with WT C57BL/6N female mice to generate heterozygous CD27<sup>+/-</sup> mice. Heterozygous F1 generation was crossed and CD27<sup>-/-</sup> homozygous mice of F2 generation used for experiments. Animals were bred and maintained under specific pathogen-free conditions at the animal facility of Hannover Medical School. We used 8–12 week old mice (sex/age matched between groups) and performed three independent experiments with groups of four to eight mice. All animal experiments were constructed according to institutional and state guidelines. The animal protocols used in this study were approved by the Committee on Animal Welfare of Lower-Saxony.

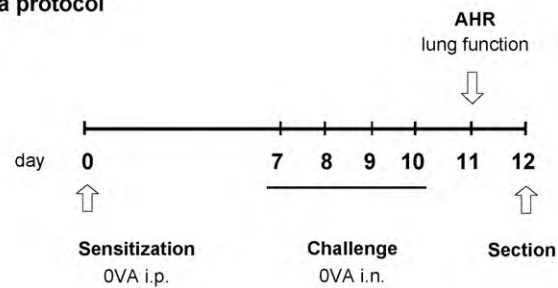
### 2.2. Immunization protocols

To induce mucosal tolerance or provoke an allergic asthma-like phenotype, mice were immunized with the model allergen Ovalbumin (OVA, Sigma–Aldrich, Steinheim, Germany). The contaminating lipopolysaccharide (LPS) in Sigma OVA (Grade V, LPS content  $\geq 1500$  EU/mg protein) was removed by Detoxi-Gel® endotoxin-removing gel columns (Pierce Biotechnology, Rockford, IL, USA) to a level  $<10$  EU/mg protein (tested with Limulus Amebocyte Lysate (LAL) Assay, Cambrex, Walkersville, MD, USA).

#### 2.2.1. Asthma protocol

Using the following immunization protocol we comparatively analyzed WT and CD27<sup>-/-</sup> mice (Fig. 1) in our murine asthma

#### (a) Asthma protocol



#### (b) Tolerance protocol

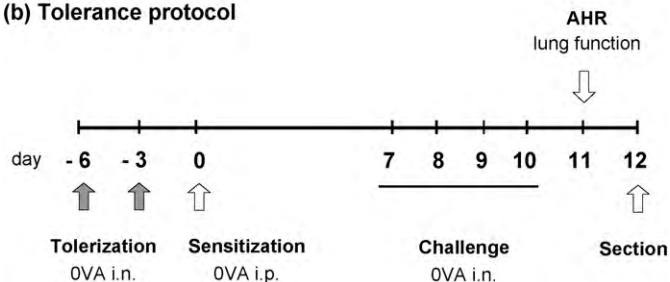


**Fig. 1.** Immunization protocols. To induce an allergic asthma phenotype (a) wild type (WT; C57BL/6) and CD27<sup>-/-</sup> (B6.129P2-CD27<sup>tm1Jbo</sup>) mice were sensitized with 20  $\mu$ g OVA in Alum Inject® i.p. on day 0, followed by intranasal challenge on consecutive days 7–10 (20  $\mu$ g OVA i.n.). One day after last OVA challenge airway hyperreactivity (AHR) was measured using a whole-body-plethysmograph. Mice were sacrificed on day 12. Control mice (“Alum”) received i.p. injections of 0.9% NaCl mixed with Alum Inject® and were challenged i.n. with 0.9% NaCl. Prevention of an asthmatic phenotype (b) was accomplished by mucosal applications of OVA (tolerization, “TOL”; 500  $\mu$ g i.n.) before sensitization (days –6 and –3).

model: mice were sensitized intraperitoneally (i.p.) with OVA (20  $\mu$ g in 200  $\mu$ l 0.9% NaCl) adsorbed to 2 mg of an aqueous solution of aluminum hydroxide and magnesium hydroxide (Imject Alum; Perbio Science, Bonn, Germany) on day 0; followed by a challenge period in which 20  $\mu$ g of OVA in 40  $\mu$ l of 0.9% NaCl was given intranasally (i.n.) on consecutive days 7–10. Control mice (“Alum”) received i.p. injections of 0.9% NaCl mixed with Imject Alum and were challenged i.n. with 0.9% NaCl. AHR was measured on day 11, and mice were sacrificed on day 12.

#### 2.2.2. Tolerance protocol

Mucosal application of OVA prior to the sensitization phase results in tolerization of mice (“TOL”) preventing the establishment of an asthmatic phenotype. Therefore we pre-treated WT and CD27<sup>-/-</sup> mice with OVA i.n. on days –6 and –3 (500  $\mu$ g OVA in 40  $\mu$ l 0.9% NaCl). Afterwards mice underwent asthma protocol as described above.

### 2.3. Measurement of airway hyperreactivity

Airway hyperreactivity (AHR) was assessed on the basis of methacholine-induced airway obstruction from conscious mice placed into whole-body plethysmograph (EMKA Technologies, Paris, France) interfaced with differential pressure transducers. Briefly, mice were exposed for 2 min to aerosolized 0.9% NaCl produced by a sonicator (model LS 290-990N), followed by increasing amounts of methacholine (20–80 mg/ml MCh) for 3 min. Enhanced pause (Penh) values were computer-assisted calculated over subsequent 4 min. Data are expressed as the percentage of the average values of baseline Penh values after 0.9% NaCl exposure (Fig. 2a; Penh curves) or values from area under the MCh-response curve analysis (Fig. 2b; AUC<sub>0–80 mg/ml</sub>).

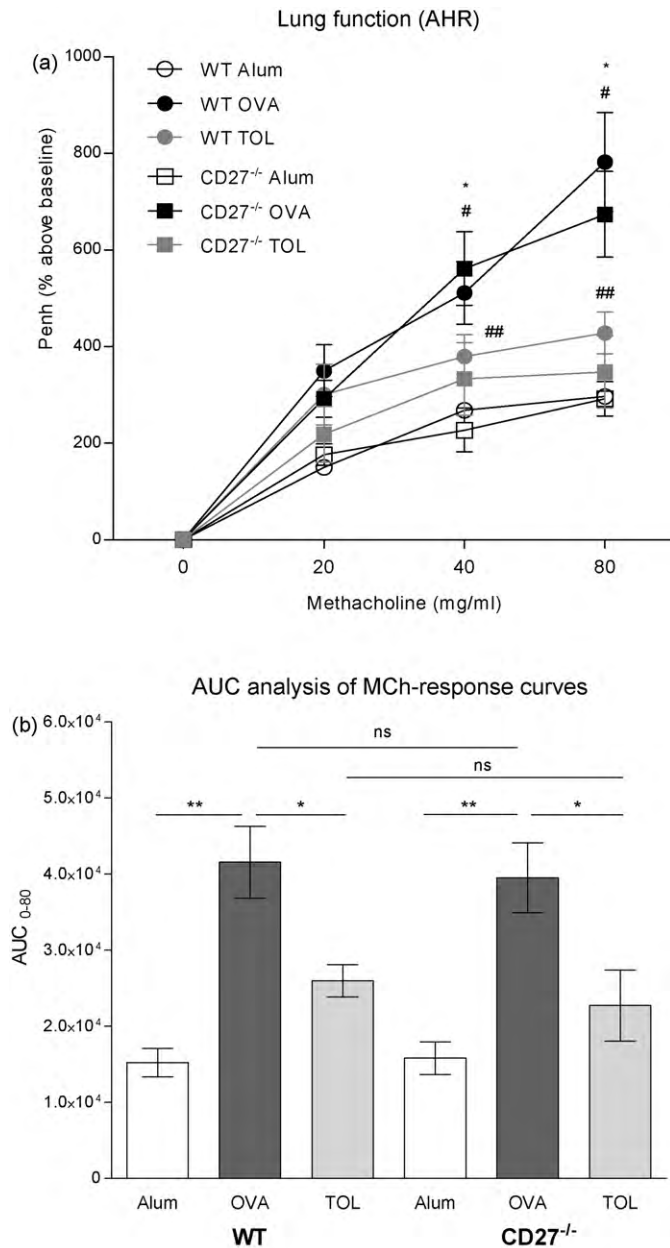


**Fig. 2.** Lung function analysis of WT and CD27<sup>-/-</sup> mice using whole-body plethysmography. AHR to methacholine challenge (MCh) was determined 24 h after last OVA challenge (day 11) by calculation of Penh values (enhanced pause). OVA sensitized and challenged WT as well as CD27<sup>-/-</sup> mice developed marked AHR compared to negative control (Alum). Tolerized mice (WT TOL; CD27<sup>-/-</sup> TOL) showed reduced level of AHR, comparable to negative control. (a) MCh-response curves. Data are shown as mean ± SEM;  $n \geq 8$ ; \* $P \leq 0.05$  WT OVA vs. corresponding Alum or TOL; CD27<sup>-/-</sup> OVA vs. corresponding Alum or TOL; # $P > 0.05$ ; WT OVA vs. CD27<sup>-/-</sup> OVA; ## $P > 0.05$ ; WT TOL vs. CD27<sup>-/-</sup> TOL; repeated measures ANOVA and Bonferroni's multiple comparison test. (b) Area under the curve (AUC) analysis of MCh-response curves. Data are shown as mean ± SEM;  $n \geq 8$ ; \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , ns  $P > 0.05$ ; One-way ANOVA and Bonferroni's multiple comparison test.

#### 2.4. Collection and analysis of bronchoalveolar lavage fluid (BALF)

During dissection of mice the trachea was cannulated and the right lung was lavaged three times with 400  $\mu$ l of PBS buffer with 2 mM EDTA to obtain a total of 1–1.2 ml BALF from each individual mouse. Afterwards BALF was centrifuged at 80  $\times$  g for 10 min and cell pellets resuspended in 500  $\mu$ l PBS/EDTA. Total number of BALF cells was identified using an automated cell counting system based on Trypan Blue exclusion method (Cedex HiRes, Innovatis,

Bielefeld, Germany). In addition, BALF differential cell counts were performed by staining cytospin slide preparations with Diff-Quick (Medion Diagnostics, Düringen, Switzerland). At least 100 cells were differentiated by light microscopy based on conventional morphologic criteria.

#### 2.5. Analysis and computer-based quantification of lung histology

The left lungs were inflated with 0.5 ml PBS and fixed in 4% formalin at RT. Harvested lungs were further embedded in paraffin, deparaffinized, cut into 4  $\mu$ m-thick sections and stained with haematoxylin and eosin (H&E, Merck, Germany) or with periodic acid–Schiff reagent (PAS, Sigma, Taufkirchen, Germany). For objective evaluation and quantification of the degree of pulmonary inflammation (H&E) and mucus production (PAS), lung sections were scanned with a digital camera (40 $\times$ ; 10 shots per lung) and analyzed with HistoClick Software (provided by I. Hohmann and W. Scherf, Germany) based on morphometric image analysis developed in our laboratory. Positive pixel counts were totalled per lung of each individual mouse and then arranged in groups for figure design and statistical analysis. The pixel data are presented as median  $\pm$  range per group of mice.

#### 2.6. Measurement of OVA-specific IgE and IgG1

Serum was obtained from each individual mouse 24 h after last i.n. OVA challenge on day 12. OVA-specific IgE and IgG1 serum levels were measured by ELISA, according to standard protocol. Using a standard curve for mouse anti-OVA IgE (AbD Serotec, Oxford, UK), we calculated OVA-specific IgE concentrations (pg/ml). Data for OVA-specific serum IgG1 are presented as titer values derived from analysis of OD values vs. factors of serum dilution series with a logarithmic curve fitting model.

#### 2.7. Measurement of in vitro cytokine production and proliferation

Cells were isolated from spleen and bLN tissue and restimulated *in vitro* ( $5 \times 10^6$  cells for spleen, for bLNs  $1 \times 10^6$  cells per well) with OVA (200  $\mu$ g/ml) in culture medium (RPMI medium supplemented with 10% FCS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). Cytokines (IL-5 and IL-13) were measured in supernatants from restimulated lymphocytes after three days by using DuoSet ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions. To determine proliferation spleen isolated cells were restimulated with 200  $\mu$ g/ml OVA in 96-well tissue-culture plates. After 48 h, cultures were pulsed with 1  $\mu$ Ci of tritiated thymidine per well for 16 h and incorporated activity was measured in a Beta-plate scintillation counter thereafter.

#### 2.8. Statistical analysis

Statistical analyses were performed using Graph Pad Prism V software (USA). First Gaussian distribution was tested by Shapiro–Wilk normality test. Data of lung function analysis (Gaussian distribution) were compared by repeated measure ANOVA and Bonferroni's post test (Fig. 2a). Additional area under the curve analysis (AUC) of MCh-response Penh curves were performed and significance tested by One-way ANOVA followed by Bonferroni's post test (Fig. 2b). For all other parameters (Figs. 3–6; non-Gaussian distribution) statistical significance between two different groups, e.g. WT OVA vs. CD27<sup>-/-</sup> OVA was estimated using Mann–Whitney–U test.  $P$  values of less than 0.05 were considered significant (\*); values of less than 0.01 as highly significant (\*\*).

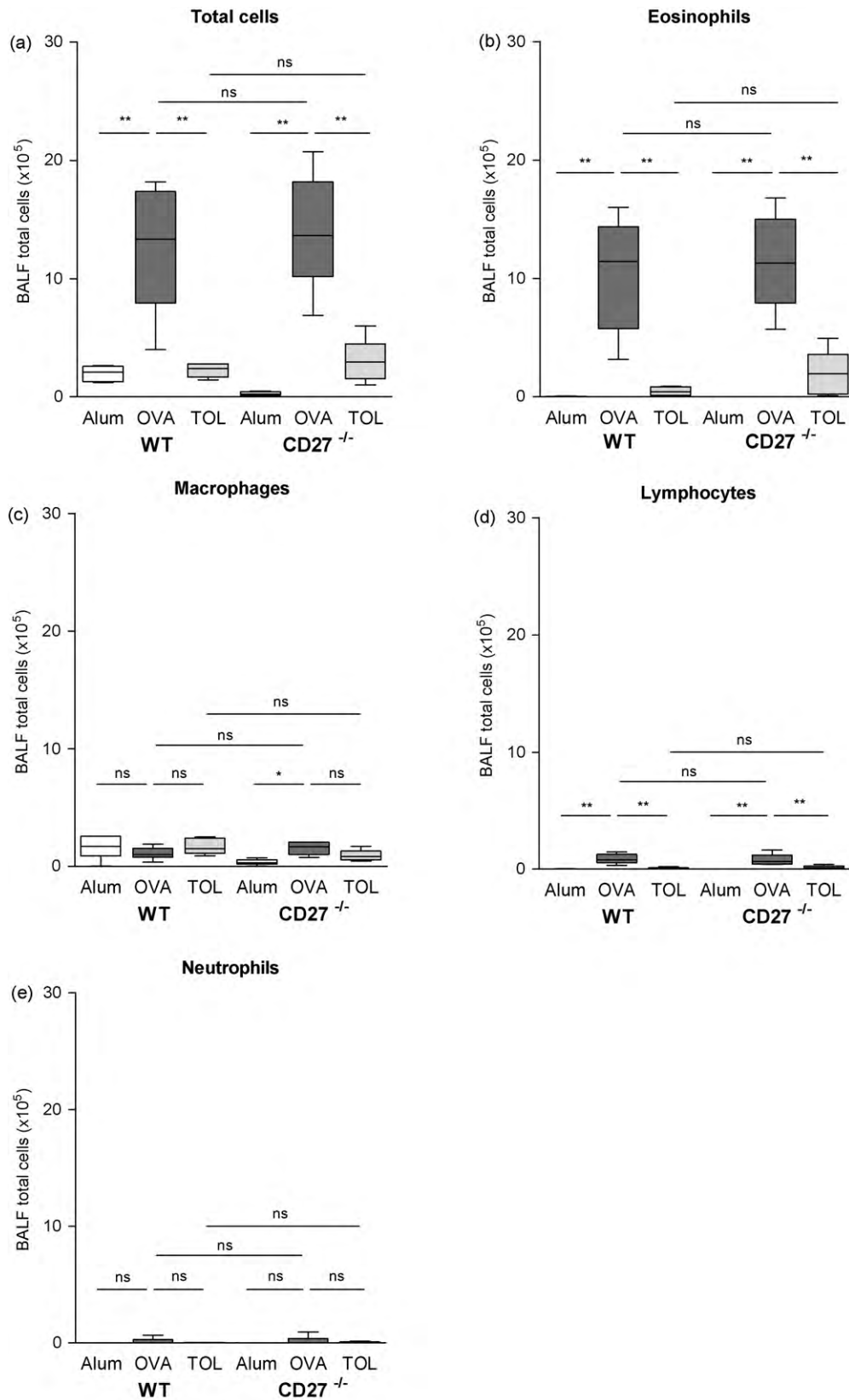


**Fig. 3.** BALF analysis of WT and CD27<sup>-/-</sup> mice. Mice were immunized with OVA according to the immunization protocol described in Fig. 1. Bronchoalveolar lavage fluid (BALF) was obtained from each individual mouse to determine total cell count (a). In addition, BALF cell differentials for eosinophils (b), macrophages (c), lymphocytes (d) and neutrophils (e) were determined on cytopins. Enhanced total cell counts (a) and eosinophilic inflammation (b) were observed in OVA sensitized and challenge WT and CD27<sup>-/-</sup> mice. In contrast, tolerant mice showed no increase in BALF total cell count and eosinophils count compared with control. Data from one representative out of three independent experiments are presented as whiskers box plots;  $n = 5-6$  animals per group; \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , ns  $P > 0.05$ ; Mann-Whitney test.

### 3. Results

#### 3.1. CD27 is not critical for the development of asthma in a murine model

##### 3.1.1. Absence of CD27 has no effect on development of AHR and allergic inflammation

We comparatively analyzed CD27 deficient (CD27<sup>-/-</sup>) vs. WT mice in our well-established murine asthma model (Fig. 1) to study whether CD27 costimulation contributes to the development of the Th2 cell driven disease allergic asthma. Naïve CD27<sup>-/-</sup> mice show normal composition of lymphoid organs, also CD4<sup>+</sup> or CD8<sup>+</sup> T cell and B cell subsets are comparable to WT [38]. As described previously [17,21,24–26], sensitization of mice by systemic and intrapulmonary application of the model allergen OVA leads to the induction of an asthmatic phenotype, characterized by AHR,

high serum IgE levels, eosinophilic lung inflammation and mucus hypersecretion.

First, we determined the effect of OVA immunization on lung function, by analyzing mice in a whole-body plethysmograph after Methacholine (MCh) challenge as shown in Fig. 2. OVA-immunized WT and CD27<sup>-/-</sup> mice, but not control mice, showed severe AHR. We did not detect a significant difference between WT OVA and CD27<sup>-/-</sup> OVA mice in regard to AHR (ns  $P > 0.05$ ).

Another major trait of allergic asthma is eosinophilic airway inflammation. Analysis of BALF revealed increased total cell counts (Fig. 3a) along with a high proportion of eosinophils (Fig. 3b) in OVA sensitized CD27<sup>-/-</sup> as well as WT mice with no significant differences between the two groups (BALF total cell counts: ns  $P = 0.699$ ; Eosinophils: ns  $P = 0.937$ ). We did not detect significant difference in levels of other BALF cell subtypes (Fig. 3c–e) between WT OVA and CD27<sup>-/-</sup> OVA mice. Next, we examined histological sections of

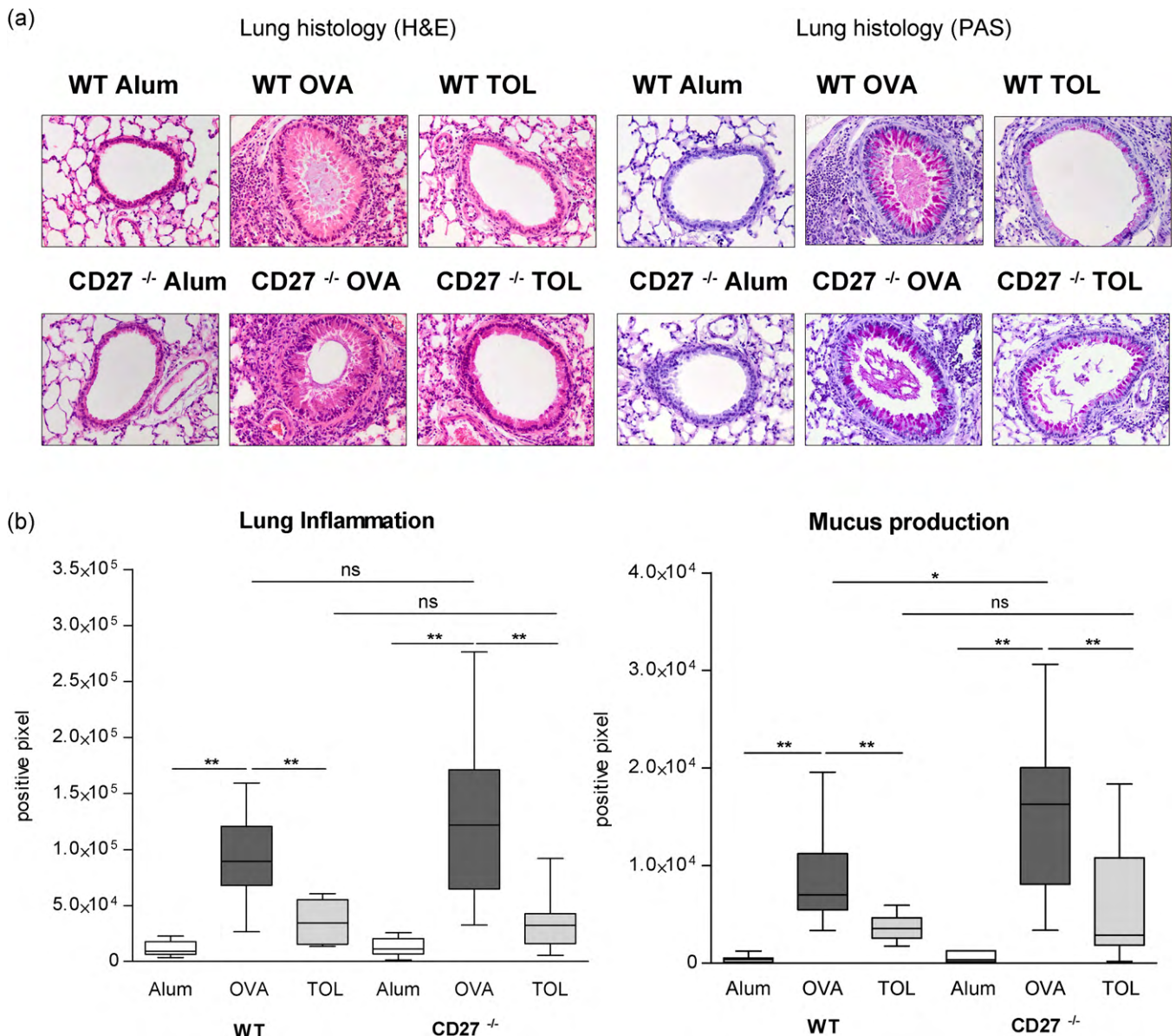


**Fig. 4.** Lung histology analysis of WT and CD27<sup>-/-</sup> mice. Lungs were obtained on day 12 for histological examination (a) after staining for inflammation (H&E, 200×; upper panel), as well as mucus (PAS, violet, 200×; lower panel), followed by computer-based quantification using an image analyzing program (b). Histological analysis revealed not only increased airway inflammation but also strong mucus production in WT OVA and CD27<sup>-/-</sup> OVA groups compared to Alum controls. Tolerance induction resulted in low levels of inflammation similar to negative controls. In comparison to CD27<sup>-/-</sup> OVA, some tolerized CD27<sup>-/-</sup> mice still showed high numbers of PAS positive regions. Data are illustrated as whiskers box plots;  $n \geq 9$ ; \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , ns  $P > 0.05$ ; Mann–Whitney test.

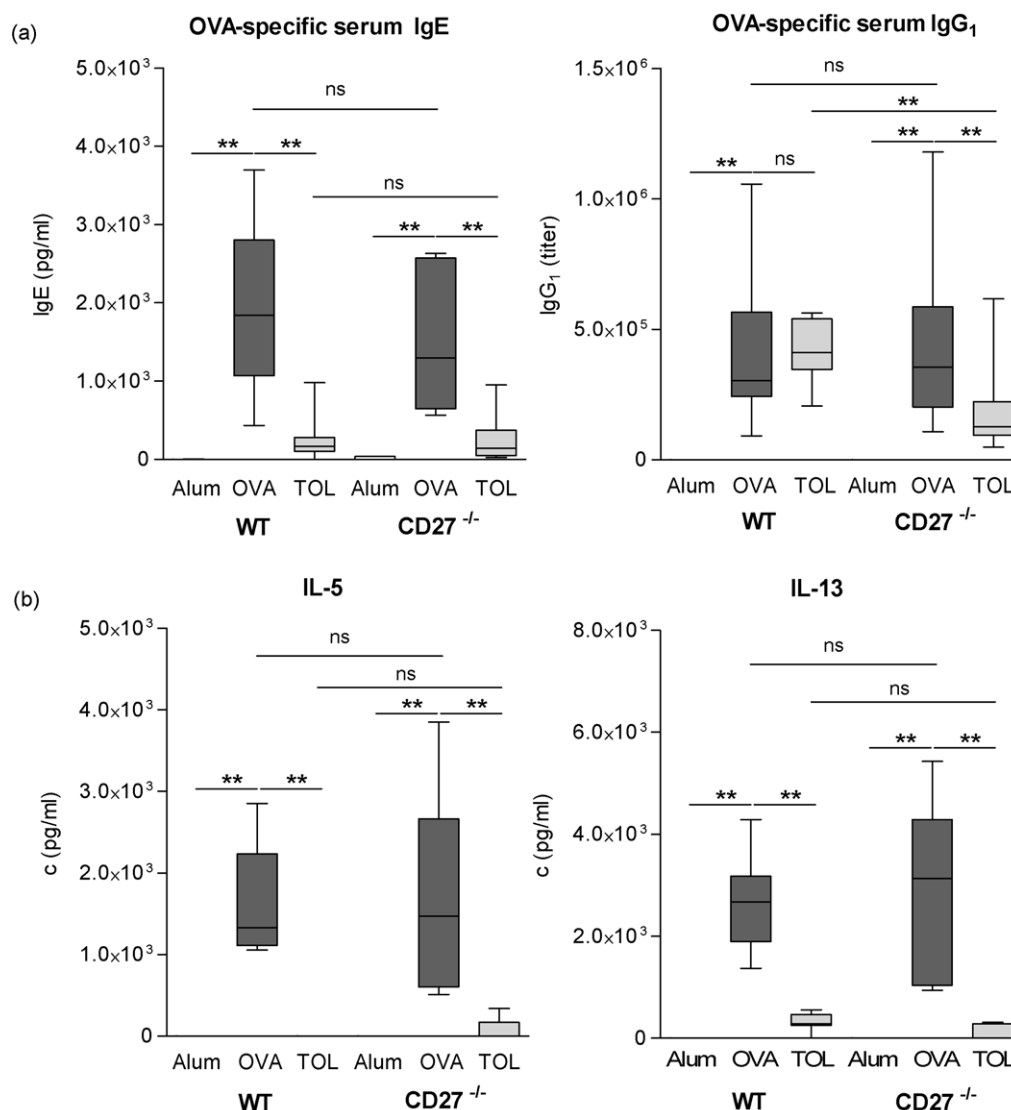


**Fig. 5.** OVA-specific serum IgE and IgG<sub>1</sub> levels in lymphocyte cell culture. OVA-specific IgE and IgG<sub>1</sub> serum levels were measured by ELISA (a). Furthermore, supernatants of pooled spleen cell cultures, restimulated *in vitro* with OVA (200 µg/ml), were analyzed by ELISA with regard to the production of the Th2 cytokines IL-5 and IL-13 (b). Data are presented as whiskers box plots, one representative out of three independent experiments is shown;  $n = 5-6$  animals per group; \*\* $P \leq 0.01$ , ns  $P > 0.05$ ; Mann-Whitney test.

lungs to determine the effect of CD27 loss on allergic lung inflammation and mucus production (Fig. 4). Like OVA sensitized WT mice, CD27<sup>-/-</sup> mice showed impressive pulmonary inflammation with perivascular and peribronchial cell infiltrates, epithelial damage and airway muscle thickening (H&E staining, Fig. 4a, left panel). This observation was confirmed by analysis of lung sections by an objective, investigator-independent computer program based on morphometric image analysis that did not reveal significant differences between the two mouse strains (ns  $P = 0.1204$ ). Using periodic acid–Schiff reagent (PAS) staining of lung slices (Fig. 4b), we found mucus hypersecretion and goblet cell hyperplasia in asthmatic WT mice, which was even exaggerated in CD27<sup>-/-</sup> OVA group (\* $P = 0.0249$ ).

### 3.1.2. CD27 deficiency has no impact on IgE serum levels, Th2 cytokine production and proliferation of splenocytes

Another feature of allergic immune response is the production of allergen-specific IgE. Hence, we determined whether the loss of CD27/CD70 interaction leads to differences in OVA-specific IgE and IgG<sub>1</sub> levels by analysis of serum via ELISA (Fig. 5a). OVA sensitization and challenge resulted in significantly increased

OVA-specific IgE levels in WT mice, whereas in corresponding negative control groups the IgE levels were very low or even undetectable (\*\* $P \leq 0.001$ ). This effect could be also observed in CD27<sup>-/-</sup> mice (\*\* $P \leq 0.001$ ). We did not identify significant changes between OVA-specific IgE serum levels of WT OVA and CD27<sup>-/-</sup> OVA animals ( $P = 0.673$ ). In regard to OVA-specific IgG<sub>1</sub> production we determined similarly enhanced levels in both OVA-treated groups compared to the negative control group (\*\* $P \leq 0.001$ ). OVA-specific IgG<sub>1</sub> levels of WT OVA and CD27<sup>-/-</sup> OVA mice were comparable (ns  $P = 0.989$ ). Levels for OVA-specific IgG<sub>2a</sub> did not differ between WT OVA and CD27<sup>-/-</sup> OVA mice (data not shown). Furthermore, we assessed cytokine production in supernatants of spleen cell cultures after *in vitro* restimulation with OVA by ELISA. In cell cultures of CD27<sup>-/-</sup> OVA and WT OVA mice we detected elevated levels of Th2 cytokines; IL-5 and IL-13 levels were markedly increased in both groups compared to Alum control (\*\* $P \leq 0.01$ ) (Fig. 5b). We did not observe significant differences between IL-5 and IL-13 production in supernatants of CD27<sup>-/-</sup> OVA mice compared to immunized WT mice (IL-5: ns  $P = 0.930$ ; IL-13: ns  $P = 1.00$ ). Besides, we did not detect a significant difference between both OVA groups in regard to Th1 response confirmed by

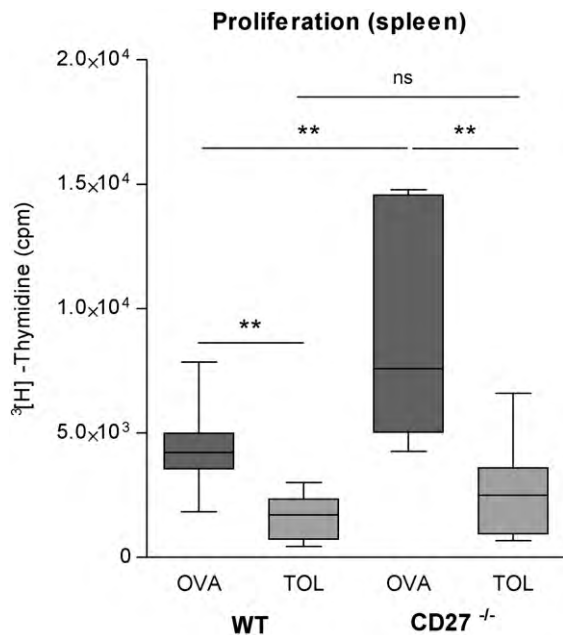


**Fig. 6.**  $^3\text{H}$ -Thymidine-proliferation assay. Splenic lymphocytes isolated from WT and CD27<sup>-/-</sup> mice were restimulated with 200  $\mu\text{g}/\text{ml}$  OVA in 96-well tissue-culture plates, pulsed with tritiated thymidine for 16 h and the incorporated activity was measured in a Betaplate scintillation counter thereafter. (a) Data from one representative out of three independent experiments are shown; median and range; \*\* $P \leq 0.01$ , ns  $P > 0.05$ ; Mann-Whitney test.

analysis of IFN- $\gamma$  levels in spleen cell culture supernatants. Results from Th1 and Th2 cytokine analysis of lung and bLN cell cultures showed comparable results (data not shown).

Moreover, we assessed *in vitro* lymphocyte proliferation after OVA restimulation by using  $^3\text{H}$ -thymidine incorporation assay (Fig. 6). Splenocytes derived from OVA sensitized WT mice showed enhanced proliferation after stimulation with increasing doses of allergen; isolated cells from CD27<sup>-/-</sup> mice showed even significantly increased proliferation with measured cpm levels even higher than WT OVA group (\*\* $P = 0.007$ ). In addition, an increased proliferation could also be determined in cultures of bLN cells isolated from both WT OVA and CD27<sup>-/-</sup> OVA mice (data not shown).

### 3.2. CD27 is not critical for the development of respiratory tolerance in a murine model

As described above we induced a comparable asthma-like phenotype in WT and CD27<sup>-/-</sup> mice by OVA sensitization and challenge. Furthermore, we were interested to see whether CD27 costimulation is involved in tolerance induction in our murine asthma model. Therefore, we tolerized mice via mucosal application of OVA prior to sensitization (Fig. 1b, tolerance protocol); in WT mice this constantly leads to the protection of the development of an asthma-like phenotype in response to OVA sensitization and challenge [27,28]. Hence, tolerized mice do not develop signs of allergic asthma which can be observed in the positive control ("OVA") and resemble the negative control group ("Alum").

Both, CD27<sup>-/-</sup> and WT mice, were equally protected from the development of an asthma-like phenotype by tolerization before start of the asthma-induction protocol. We did not detect significant differences between tolerized WT and CD27<sup>-/-</sup> mice with regard to AHR (Fig. 2; ns  $P > 0.05$ ), total cell and eosinophils count in the BALF (Fig. 3; ns  $P = 0.937$ ), pulmonary inflammation in H&E stained lung sections (Fig. 4a; ns  $P = 0.780$ ), mucus production in PAS stained lung sections (Fig. 4b; ns  $P = 0.720$ ), OVA-specific IgE (Fig. 5a; IgE: ns  $P = 0.704$ ) or IgG<sub>2a</sub> serum levels (data not shown).

Moreover, *in vitro* proliferation and Th2 cytokine production after OVA restimulation of splenocytes from tolerized CD27<sup>-/-</sup> and WT mice were equally reduced compared to the corresponding OVA sensitized mice that were not tolerized (Fig. 6; proliferation spleen: ns  $P = 0.127$ ; Fig. 5b; cytokine production: IL-5: ns  $P = 1.000$ ; IL-13: ns  $P = 0.072$ ). In addition, we observed comparable IFN- $\gamma$  levels in spleen cell culture supernatants from both tolerized CD27<sup>-/-</sup> and WT mice (data not shown). Only one parameter differed significantly between both mice strains (Fig. 5a): Tolerized WT mice had high IgG<sub>1</sub> levels that were comparable to OVA sensitized WT mice (ns  $P = 0.9887$ ). In contrast, the OVA-specific IgG<sub>1</sub> levels in tolerized CD27<sup>-/-</sup> animals were significantly lower as in the corresponding OVA group (\*\* $P \leq 0.001$ ) and in the corresponding tolerized WT group (\*\* $P = 0.0014$ ). However, all other measured parameters were comparable in tolerized WT and CD27<sup>-/-</sup> mice demonstrating that CD27 costimulation does not play a crucial role in respiratory tolerance induction in our murine model.

## 4. Discussion

Here, we demonstrate in a murine model of asthma that absence of CD27 signalling in CD27<sup>-/-</sup> mice does not affect the development of this Th2 cell dominated disease. We observed strong AHR, profound airway eosinophilic inflammation as well as mucus hypersecretion, enhanced OVA-specific serum IgE levels, in both OVA-immunized WT and CD27<sup>-/-</sup> mice. Mucus production of CD27<sup>-/-</sup> OVA mice seemed to be even higher compared to WT animals, however IL-13 levels in spleen cell culture supernatants did not differ between both OVA groups as did other Th2 cytokine levels. The only significant difference between CD27<sup>-/-</sup> and WT mice was a stronger proliferation in splenocytes derived from CD27<sup>-/-</sup> OVA mice.

Our results go in line with a study about the role of CD27 for the development of allergic eye inflammation. Using an experimental model for allergic conjunctivitis (EC) Sumi et al. showed that inhibition of CD27/CD70 costimulation by treatment with monoclonal antibodies did not significantly alter ocular eosinophilic infiltration in mice; hence modulation of CD27/CD70 signalling had no effect on EC severity [15]. In contrast to these findings in two different allergy models, other costimulatory molecules of the TNFR family like CD30, CD134 (OX40) or CD137 (4-1BB) have been shown to be critically involved in the development of asthma [16,17,20–22] and EC [29,30].

In asthma, prophylactic administration of an agonistic CD137 mAb was capable of preventing the establishment of AHR, eosinophilic infiltration and production of allergen-specific IgE and reduced the production of Th2 cytokines while enhancing secretion of Th1 cytokines. Depletion of CD8<sup>+</sup> T cells or blockade of IFN- $\gamma$  abolished the protective effect of 4-1BB antibody, which suggests that the effect of anti-4-1BB antibody was dependent on IFN- $\gamma$ -producing CD8<sup>+</sup> T cells. Injection of anti-4-1BB antibody after sensitization totally reversed the allergic phenotype in mice, which suggests that intervention within the 4-1BB pathway might offer a novel therapeutic approach in patients with asthma [21]. In parallel, in a similar approach using a model of atopic conjunctivitis, treatment with an agonistic anti-4-1BB before or after sensitization abolished the development of allergic conjunctivitis [29]. For CD30, several studies have shown indirect evidence for a role in allergic diseases by correlating CD30 expression and sCD30 levels to severity and outcome of allergic asthma and atopic dermatitis [31–33]. By using CD30<sup>-/-</sup> mice and mAbs directed against CD30 receptor or ligand, we could directly demonstrate a critical role of CD30 signalling in the development of asthma [16,17]. Similarly, OX40- or OX40L-deficient mice exhibit markedly impaired reactivation of Th2 memory cells and Th2 responses as well as diminished lung inflammation [18,19,34]. In models of allergic lung inflammation

OX40-deficient mice developed a weak Th2 response and airway inflammation after sensitization to OVA indicating the cardinal role of this molecule in the initiation of allergic immune responses [19]. Moreover, OX40/OX40L as well as 4-1BB/4-1BBL costimulation are discussed to be important regulators of Treg responses [35] and critical involved in the modulation of tolerance [36,37]. However, our analysis of CD27<sup>-/-</sup> mice in a respiratory tolerance model showed that CD27 signalling is not essential for the development of respiratory tolerance, since CD27 deficient mice were equally protected from the development of asthma as WT mice.

While we and others did not find an important role of CD27 for the CD4<sup>+</sup> mediated allergic diseases asthma and EC [15], several studies indicate a strong contribution of CD27/CD70 costimulation to CD8<sup>+</sup> mediated T cell responses [9]. CD27 appears to promote the survival of virus-specific CD8<sup>+</sup> T cells by allowing successive T cell divisions, rather than directly affecting cell cycle entry or activity [38,39]. Interestingly, although Hendriks et al. observed reduced T cell accumulation at site of infection in CD27<sup>-/-</sup> mice, there were no differences in regard to influenza severity or recovery time period detectable between WT and CD27<sup>-/-</sup> mice. Moreover, the lack of CD27 did not affect the cytolytic activity of CD8<sup>+</sup> T cells nor had an impact on the B cell response to influenza virus. However, analysis of CD70tg mice in an influenza virus model supports the importance of CD27/CD70 interaction for CD8<sup>+</sup> T cell accumulation [40] and application of soluble recombinant CD70 OVA peptide immunization increases the size of OVA-specific CD8<sup>+</sup> T effector and memory cell pool in vivo [41]. Furthermore, the stimulatory effect of CD27/CD70 signalling on CD8<sup>+</sup> T effector cell responses could also be observed in a cardiac allograft model [42], in which CD70 inhibition resulted in graft survival mediated by the inhibition of TNF- $\alpha$  production. In this study CD4<sup>+</sup> T cell accumulation and function were less affected.

Our data together with the above mentioned studies suggest that CD27/CD70 costimulation may be preferentially involved in CD8<sup>+</sup> rather than CD4<sup>+</sup> cell mediated immune reactions. CD27/CD70 interaction is further discussed to be more involved in CD4<sup>+</sup> Th1-mediated disease rather than CD4<sup>+</sup> Th2 responses. This idea is supported by results of Nakajima et al. indicating a role of CD27/CD70 interaction in the induction of autoimmune disease [43]. Treatment of mice with anti-CD70 mAb prevented the development of Th1-mediated experimental autoimmune encephalomyelitis (EAE) due to the inhibition of TNF- $\alpha$  production. Recently published data of Oflazoglu underlines the therapeutic potentiality of anti-CD70 treatment in autoimmune diseases [44]. CD70 inhibition resulted in reduced production of autoantibody, decrease of inflammation and bone and cartilage destruction, leading to marked improvement of clinical symptoms in a murine model for rheumatoid arthritis. Moreover, anti-CD70 treatment was effective in prevention and treatment of Th1-mediated colitis in a mouse model [45].

Taken together, we conclude that CD27/CD70 costimulation does not play a crucial role in the development of an asthma-like phenotype in our murine asthma model. Thus, we hypothesize that CD27 signalling is dispensable for Th2-mediated allergic immune responses and seems to be no target for asthma therapy.

## Conflict of interest

The authors declare no financial or commercial conflict of interest.

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