

## CD28/CTLA4 double deficient mice demonstrate crucial role for B7 co-stimulation in the induction of allergic lower airways disease

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### Summary

**Background** The existence of a third B7-1/B7-2 receptor was postulated in a recent study using a novel mouse strain lacking both CD28 and CTLA4 (CD28/CTLA4<sup>-/-</sup>).

**Objective** In the present study, it was investigated if T cell co-stimulation *via* the putative B7-1/B7-2 receptor plays a role in the induction of Th2-mediated asthma manifestations in mice.

**Methods** BALB/c wild-type, CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice were sensitized and aerosol challenged with ovalbumin (OVA).

**Results** At 24 h after the last aerosol, wild-type mice showed airway hyper-responsiveness *in vivo* and up-regulated levels of serum OVA-specific IgE compared with the situation shortly before OVA challenge. In addition, eosinophil numbers and IL-5 levels in the broncho-alveolar lavage fluid and Th2 cytokine production by lung cells upon OVA re-stimulation *in vitro* were observed. In agreement with an earlier study, we failed to induce any of the asthma manifestations in B7-1/B7-2<sup>-/-</sup> mice. Importantly, also CD28/CTLA4<sup>-/-</sup> mice showed no asthma manifestations upon OVA sensitization and challenge.

**Conclusion** These data clearly demonstrate that T cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated asthma manifestations in this murine model and, conversely, that CD28 signalling is crucial.

**Keywords** airway hyper-responsiveness, allergy, asthma, co-stimulation, eosinophilia, immunoglobulin E, T lymphocytes

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### Introduction

Allergic asthma is a complex inflammatory disease of the airways characterized by reversible airflow obstruction, persistent airway hyper-responsiveness to bronchospasmogenic stimuli such as methacholine, chronic eosinophilic airway inflammation and airway remodelling [1]. Allergen-specific CD4<sup>+</sup> type 2 helper T (Th2) cells play a pivotal role in the pathogenesis and progression of allergic asthma by orchestrating the inflammatory response [2]. Over the past decade, it is well established that for optimal activation, T lymphocytes require nonspecific co-stimulatory signals in addition to the antigen-specific signal conferred by the T cell receptor (TCR) [3–5]. Although many receptor-ligand pairs for T cell co-stimulation have been identified since, CD28 is the primary co-stimulatory molecule constitutively present on the surface of T cells. Engagement of CD28 on naïve T cells by its ligands B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells provides a potent co-stimulatory signal to T

cells activated through their TCR that contributes to elements of T cell activation such as proliferation and IL-2 secretion. The second receptor for the B7-1/B7-2 ligands, CTLA4, is expressed on activated T cells and delivers an inhibitory signal to terminate the T cell response [6]. In experimental murine models of allergic asthma, blockade of the CD28/CTLA4:B7-1/B7-2 pathway of T cell co-stimulation during the antigen sensitization and/or challenge period ameliorates the features of allergic airway sensitization [7–12].

Interestingly, a recent study using a novel mouse strain lacking both CD28 and CTLA4 (CD28/CTLA4<sup>-/-</sup>) provided evidence for the existence of an additional receptor for the B7-1 and B7-2 molecules [13]. In this study, CD28/CTLA4<sup>-/-</sup> CD4<sup>+</sup> T cells demonstrated considerable residual B7-1/B7-2-dependent proliferation upon polyclonal stimulation *in vitro*. In addition, T cell co-stimulation *via* this putative B7-1/B7-2 receptor was shown to contribute substantially to Th1-mediated cardiac allograft rejection *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2<sup>-/-</sup> mice [13–15].

The role of this putative B7-1/B7-2 receptor in other T cell-mediated disease models is unknown at the moment. With regard to Th2-mediated allergic asthma, no features of allergic airway sensitization can be induced in B7-1/B7-2<sup>-/-</sup>

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mice [16] nor in wild-type mice treated with mAbs against both B7-1 and B7-2 throughout the antigen sensitization and challenge period [17]. In CD28<sup>-/-</sup> mice, however, the failure to induce manifestations of allergic asthma is accompanied by the presence of lymphocytes and IFN- $\gamma$  in the bronchoalveolar lavage fluid (BALF) [17, 18] and the priming of lymphocytes in the lungs, spleen and lymph nodes [17–19]. These data on residual T cell activation in CD28<sup>-/-</sup> mice, but not in B7-1/B7-2<sup>-/-</sup> mice [16], suggest a potential role for the putative B7-1/B7-2 receptor. Co-stimulation by the supposed B7-1/B7-2 receptor would then result in allergen-specific T cell activation that is not strong enough to induce the features of allergic airway sensitization. However, the failure to induce these symptoms in CD28<sup>-/-</sup> mice might also be due to negative signalling by CTLA4 [20–22] that opposes potential T cell co-stimulation *via* the putative B7-1/B7-2 receptor. So, unmasking the possible role of co-stimulation by the supposed B7-1/B7-2 receptor in T cell activation requires the absence of both CD28 and CTLA4.

Therefore, in the present study, we investigated if T cell co-stimulation *via* the putative B7-1/B7-2 receptor contributes to the induction of the Th2-dominated model of allergic asthma using the novel CD28/CTLA4<sup>-/-</sup> mouse strain. To this end, BALB/c wild-type mice, CD28/CTLA4<sup>-/-</sup> mice and B7-1/B7-2<sup>-/-</sup> mice were sensitized and aerosol challenged with the experimental allergen ovalbumin (OVA). At 24 h after the last OVA aerosol, wild-type mice show airway hyper-responsiveness to the non-specific stimulus methacholine, allergen-specific IgE in serum and an infiltration of eosinophils in the BALF. In addition, Th2-type cytokines were observed in the BALF and supernatant of OVA re-stimulated lung cell cultures. In agreement with an earlier study by Mark et al. [16], we failed to induce any of these features of allergic airway sensitization in the B7-1/B7-2<sup>-/-</sup> mice. Interestingly, also CD28/CTLA4<sup>-/-</sup> mice showed no manifestations of allergic asthma upon OVA sensitization and challenge. In addition, we were unable to demonstrate signs of residual OVA-specific CD4<sup>+</sup> T cell activation in CD28/CTLA4<sup>-/-</sup> mice. So, T cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated features of allergic airway sensitization in this murine model. Conversely, further evidence is provided for the critical role of CD28 signalling in the development of Th2-dominated allergic inflammatory responses [17, 19, 23].

## Materials and methods

### Animals

Specific pathogen-free wild-type BALB/c mice were obtained from Charles River (Maastricht, the Netherlands). BALB/c CD28/CTLA4<sup>-/-</sup> mice [13] and BALB/c B7-1/B7-2<sup>-/-</sup> mice [24] were generated in the laboratory of Dr A. H. Sharpe (Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA), which is an institution accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC). In the Netherlands, these mice were bred and maintained in a pathogen-free animal facility of the Central Laboratory Animal Institute (Utrecht).

Female and male (4:2) 12–13-week-old mice were used for our studies. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments.

### Antigen sensitization and challenge

All mice were sensitized and challenged with OVA (chicken egg albumin, crude grade V, Sigma, St Louis, MO, USA). Systemic sensitization was performed by two i.p. injections of 10  $\mu$ g OVA adsorbed onto 2.25 mg aluminium hydroxide (alum; ImjectAlum, Pierce, Rockford, IL, USA) in 0.1 mL pyrogen-free saline on days 0 and 7. On days 35, 38 and 41, mice were challenged by inhalation of an OVA aerosol in a Plexiglass exposure chamber (5 L) for 20 min. The aerosols were generated by nebulizing an OVA solution (10 mg/mL in pyrogen-free saline) using a Pari LC Star nebulizer (PARI Respiratory Equipment, Richmond, VA, USA; particle size 2.5–3.1  $\mu$ m) driven by compressed air at a flow rate of 6 L/min.

### Measurement of airway responsiveness *in vivo*

Airway responsiveness was measured in conscious, unrestrained mice on day 30 (5 days before the OVA aerosol challenge period) and at 24 h after the last OVA aerosol challenge on day 42, by recording respiratory pressure curves in response to inhaled nebulized methacholine (acetyl- $\beta$ -methylcholine chloride; Sigma) using barometric whole-body plethysmography (Buxco; EMKA Technologies, Paris, France). Airway responses were expressed in enhanced pause ( $P_{\text{enh}}$ ), an index of airway obstruction as described in detail previously [25]. Briefly, mice were placed in a whole-body chamber and pressure differences between this chamber and a reference chamber were recorded. After baseline  $P_{\text{enh}}$  values were obtained for 3 min and averaged, animals were exposed to a saline aerosol and a series of methacholine aerosols (solutions doubling in concentration, ranging from 1.56 to 50 mg/mL in saline). Aerosols were generated by a Pari LC Star nebulizer for 3 min and after each nebulization, readings were taken for 3 min and averaged.

### Determination of OVA-specific IgE levels in serum

At days -3 and 32 (3 days before challenge), approximately 0.25 mL blood was recovered from mice by an incision in the tail vein. After measurement of airway responsiveness *in vivo* at 24 h after the last OVA aerosol challenge on day 42, mice were killed by i.p. injection of 1 mL 10% urethane (Sigma) in sterile saline and bled by cardiac puncture. Serum was prepared from blood samples and stored at -20 °C until determination of OVA-specific IgE levels by ELISA. Briefly, Maxisorp 96-wells flat-bottomed microtitre plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4 °C with 1  $\mu$ g/mL rat anti-mouse IgE mAb (clone R35-72, PharMingen, San Diego, CA, USA) diluted in PBS. The next day, the ELISA was performed at room temperature using ELISA buffer (PBS containing 0.5% BSA [Sigma], 2 mM EDTA, 136.9 mM NaCl, 50 mM Tris, 0.05% Tween-20 [Merck, Whitehouse Station, NJ, USA], pH 7.2) for blocking and dilution of samples, standard and reagents and PBS containing 0.05% Tween-20 for washing between incubations. After blocking of wells for 1 h, serum samples and a duplicate dilution series of

an OVA-specific reference serum (starting 1:40) were added to the wells and incubated for 2 h. An OVA-specific IgE reference serum was obtained by sensitization and challenge of mice with OVA as described above and arbitrarily assigned a value of 1000 experimental units/mL (EU/mL). Hereafter, 1 µg/mL of OVA labelled to digoxigenin (DIG) by a DIG protein labelling kit (Roche Diagnostics, Basel, Switzerland) was added for 1.5 h, followed by incubation with 1:500 diluted anti-DIG-Fab fragments coupled to horseradish peroxidase (Roche Diagnostics) for 1 h. Colour development was performed with *o*-phenylenediamine-dichloride substrate (0.4 mg/mL, Sigma) and 4 mM H<sub>2</sub>O<sub>2</sub> in PBS and the reaction was stopped by adding 4 M H<sub>2</sub>SO<sub>4</sub>. The OD was read at 490 nm, using a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Results were analysed with Microplate Manager PC software (Bio-Rad Laboratories). The lower detection limit of the ELISA was 4 EU/mL IgE.

#### Analysis of the BALF

BAL was obtained immediately after bleeding of the mice by lavage of the airways through a tracheal cannula with 1 mL saline at 37 °C containing 5% BSA and 2 µg/mL aprotinin (Roche Diagnostics). The recovered lavage fluid of this first ml was kept apart for determination of the amount of IL-5 in the supernatant by ELISA. Subsequently, mice were lavaged four times with 1 mL aliquots of saline only at 37 °C. Recovered lavage fluid of the second through fifth ml was pooled and cells (including those from the first ml) were pelleted (387 g, 4 °C, 10 min), and resuspended in 0.15 mL cold PBS. The total number of cells in the BALF was determined using a Bürker–Türk counting-chamber (Karl Hecht Assistant KG, Sondheim/Röhm, Germany). For differential BALF cell counts, cytospin preparations were made (15 g, 4 °C, 5 min) using a cytocentrifuge (Shandon Life Science, Cheshire, UK), and cells were fixed and stained with Diff-Quick (Dade AG, Düringen, Switzerland). Per cytospin, at least 200 cells were counted and differentiated into mononuclear cells (monocytes, macrophages and lymphocytes), eosinophils and neutrophils by standard morphology and staining characteristics. The investigator counting the cells was blinded to the treatment groups.

#### Culture of lung cells

Lungs were lavaged as described above and perfused *via* the right heart ventricle with 4 mL saline at 37 °C containing 100 U/mL heparin (Leo Pharmaceuticals, Weesp, the Netherlands) to remove any blood and intravascular leucocytes. Complete lung tissue was removed and transferred to cold sterile PBS. Lungs were then minced and digested in 3 mL RPMI 1640 containing 2.4 mg/mL collagenase A and 1.0 mg/mL DNase I (grade II) (both from Roche Diagnostics) for 30 min at 37 °C. Enzyme activity was stopped by adding 1 mL FCS. The lung tissue digest was filtered through a 70-µm nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 10 mL RPMI 1640 to obtain a single-cell suspension. The lung-cell suspension was washed, resuspended in culture medium (RPMI 1640 supplemented with 10% FCS, 1% glutamax I and gentamicin [both from Life Technologies, Gaithersburg, MD, USA] and

50 µM β-mercaptoethanol [Sigma]) and the total number of lung cells was determined using a Bürker–Türk counting-chamber. Lung cells (8 × 10<sup>5</sup> lung cells/well) were cultured in 96-well round-bottomed plates (Greiner Bio-One, Kremsmuenster, Austria) in the presence or absence of OVA (10 µg/mL). In addition, lung cells were polyclonally stimulated with plate-bound rat anti-mouse CD3 mAb (clone 17A2 rIgG2b, 50 µg/mL, coated overnight at 4 °C). The hybridoma for the 17A2 mAb to CD3 was obtained from the American Type Tissue Collection (Manassas, VA, USA) and purified Ab was used. After 5 days of culture at 37 °C in 5% CO<sub>2</sub>, the supernatant was harvested, and stored at –20 °C until determination of cytokine levels by ELISA.

#### Measurement of cytokines

Supernatants of the first millilitre BALF and of the lung cell cultures were analysed for IFN-γ, IL-5 and IL-10 content by sandwich ELISA using Ab pairs and standards purchased from PharMingen, according to the manufacturer's instructions. The lower detection limits of the ELISAs were 16 pg/mL for IFN-γ, 32 pg/mL for IL-5 and 63 pg/mL for IL-10.

#### Statistical analysis

All data are expressed as mean ± SEM. The airway dose-response curves to methacholine were statistically analysed by a general linear model of repeated measurements followed by *post hoc* comparison between groups. Data were log<sub>10</sub> transformed before analysis to equalize variances in all groups. Statistical analysis on BALF cell counts was performed using the non-parametric Mann–Whitney *U*-test. For all other parameters, results were statistically analysed using a Student's *t*-test (two-tailed, homoscedastic). Differences between groups were considered statistically significant at the *P* < 0.05 level. Statistical analyses were performed using SPSS for Windows version 10.0.5 (SPSS, Chicago, IL, USA).

## Results

BALB/c wild-type, CD28/CTLA4<sup>–/–</sup> and B7-1/B7-2<sup>–/–</sup> mice were i.p. sensitized to 10 µg OVA adsorbed to alum on day 0 and 7 and aerosol challenged with a 1.0% OVA solution for 20 min on days 35, 38 and 41.

#### Absence of OVA-specific IgE in the sera from CD28/CTLA4<sup>–/–</sup> and B7-1/B7-2<sup>–/–</sup> mice

To determine allergen-specific IgE levels in serum, blood was withdrawn from each mouse before sensitization to OVA (pre-serum), after sensitization but before the OVA aerosol challenge period (post-sensitization serum) and at 24 h after the last OVA aerosol (final serum). In the pre-sera from the three BALB/c strains, no OVA-specific IgE could be detected. After sensitization to OVA, antigen-specific IgE was found in the sera from wild-type mice (11,664 ± 2,034 EU/mL) and these levels were greatly up-regulated upon OVA aerosol challenge (103,487 ± 15,811 EU/mL, *P* < 0.0005). In contrast, in both the post-sensitization- and final sera from CD28/

CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice, the amount of OVA-specific IgE was below the detection level of the ELISA (4 EU/mL).

These data suggest that no sensitization to OVA had taken place in the animals deficient in CD28 and CTLA4 or the B7 molecules.

#### *Absence of airway hyper-responsiveness in CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice*

Airway responsiveness *in vivo* to methacholine was determined in conscious, unrestrained mice by barometric whole-body plethysmography and was first measured after sensitization to OVA, but before the aerosol challenge period. As shown in Fig. 1, all three OVA-sensitized BALB/c strains demonstrated a dose-dependent increase in  $P_{\text{enh}}$ , an index of airway obstruction, in response to aerosolized methacholine and the complete methacholine dose-response curves (DRCs) did not differ between the strains ( $P = 1$  for each combination of two strains). Airway responsiveness *in vivo* was measured again at 24 h after the last OVA aerosol. Upon OVA aerosol challenge, wild-type mice developed airway hyper-responsiveness as the second methacholine DRC is significantly different from the DRC obtained before challenge in this group of mice ( $P < 0.05$ ). In contrast, airway responsiveness to methacholine before- and at 24 h after the OVA aerosol challenge period was not different in CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice, when comparing the complete DRCs ( $P = 1$  and 1, respectively). Comparing the airway responses to the separate concentrations of the methacholine DRC before and after OVA challenge, CD28/CTLA4<sup>-/-</sup> mice showed airway hyper-responsiveness to 50 mg/mL methacholine ( $P < 0.05$ , Fig. 1). However, in a duplicate experiment, CD28/CTLA4<sup>-/-</sup> mice demonstrated no induction of hyper-responsiveness upon OVA challenge to each separate concentration of the methacholine DRC (data not shown).

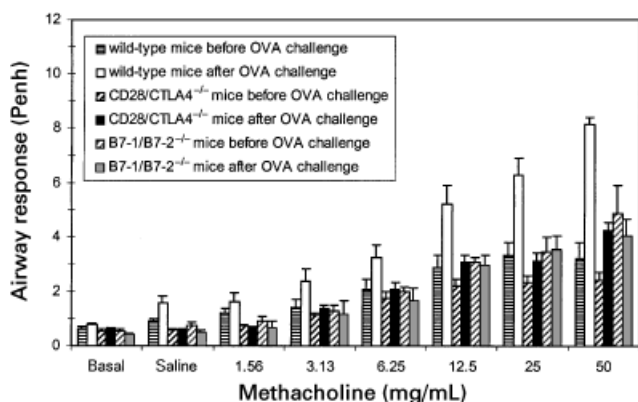


**Fig. 1.** Absence of airway hyper-responsiveness in CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice. BALB/c wild-type (first and second bar), CD28/CTLA4<sup>-/-</sup> (third and fourth bar) and B7-1/B7-2<sup>-/-</sup> mice (fifth and sixth bar) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. Airway responsiveness *in vivo* to aerosolized methacholine was measured in conscious, unrestrained mice by whole-body plethysmography before (striped bars) and at 24 h after (plain bars) the OVA aerosol challenge period. Values are expressed as mean  $\pm$  SEM ( $n = 5-6$  per group).

So, no residual airway hyper-responsiveness was induced in mice lacking both CD28 and CTLA4 upon OVA-sensitization and challenge.

#### *Absence of eosinophils and IL-5 in the BALF from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice*

The number of various leucocytes in the BALF was used as a measure for the infiltration of these cells in the airways and was determined at 24 h after the last OVA aerosol challenge. OVA sensitization and challenge from wild-type mice resulted in an influx of inflammatory cells in the BALF, predominantly eosinophils besides mononuclear cells (monocytes, macrophages and lymphocytes) and a few neutrophils (Fig. 2). In contrast, as in naïve wild-type mice (data not shown), only mononuclear cells were present in the BALF from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice upon OVA-sensitization and challenge and the number of mononuclear cells was significantly less compared with OVA-sensitized and challenged wild-type mice ( $P < 0.005$  and  $< 0.05$ , respectively).

The influx of inflammatory cells in the BALF of OVA-sensitized and challenged wild-type mice was accompanied by the presence of IL-5 ( $0.67 \pm 0.08$  ng/mL), but no detectable levels of IFN- $\gamma$ , pointing to a Th2-dominated response. Because IL-5 is involved in the migration of eosinophils, this is in accordance with the BALF eosinophilia observed in this group of mice. In contrast, no IL-5 (and no IFN- $\gamma$ ) could be detected in the BALF from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice.

So, no residual Th2-dominated inflammatory response could be observed in the BALF from CD28/CTLA4<sup>-/-</sup> mice upon OVA sensitization and challenge.

#### *Lung T cells from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice are not primed*

A single-cell suspension of lung tissue was prepared at 24 h after the last OVA aerosol. To determine antigen-specific T cell cytokine responses, lung cells were re-stimulated with

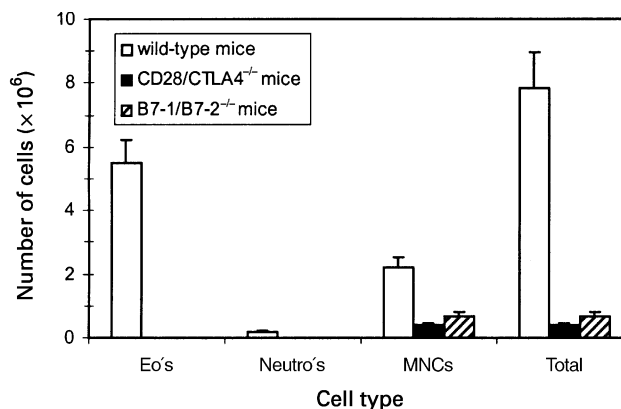


**Fig. 2.** Absence of eosinophils in the BALF from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice. BALB/c wild-type (open bars), CD28/CTLA4<sup>-/-</sup> (closed bars) and B7-1/B7-2<sup>-/-</sup> mice (hatched bars) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, BALF was recovered and the cellular composition was determined. Values are expressed as mean  $\pm$  SEM ( $n = 5-6$  per group).

10 µg/mL OVA for 5 days. In addition, lung T cells were polyclonally stimulated with 50 µg/mL anti-CD3 mAb to determine their intrinsic capacity to produce cytokines or cultured with medium only.

Lung cell cultures derived from OVA-sensitized and challenged wild-type mice only produced a small amount of IFN-γ upon stimulation with anti-CD3 mAb ( $0.042 \pm 0.014$  ng/mL, Fig. 3) and this amount was reduced compared with cultures from naïve wild-type mice ( $0.128 \pm 0.021$  ng/mL, data not shown in Fig. 3). However, these cultures produced fair amounts of the Th2 cytokines IL-5 and IL-10 upon re-stimulation with OVA ( $8.20 \pm 1.35$  and  $4.73 \pm 1.48$  ng/mL,

respectively) and polyclonal stimulation ( $7.97 \pm 1.45$  and  $3.31 \pm 0.71$  ng/mL, respectively). So, OVA-specific T cells were present in lung tissue derived from OVA-sensitized and challenged wild-type mice that showed a clear Th2 response upon antigenic re-stimulation *in vitro*.

Polyclonal stimulation of lung cell cultures derived from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice sensitized and challenged with OVA, resulted in significantly more IFN-γ production ( $0.52 \pm 0.09$  ng/mL,  $P < 0.005$  and  $0.39 \pm 0.13$  pg/mL,  $P < 0.05$ , respectively) compared with cultures from OVA-sensitized and challenged wild-type mice ( $0.042 \pm 0.014$  ng/mL). In contrast, significantly less IL-10 could be observed in the supernatant of these cultures after 5 days of stimulation with anti-CD3 mAb ( $1.09 \pm 0.12$  ng/mL,  $P < 0.01$  and  $0.69 \pm 0.17$  pg/mL,  $P < 0.01$ , respectively), compared with those from OVA-sensitized and challenged wild-type mice ( $3.31 \pm 0.71$  ng/mL). IL-5 could only be detected in the supernatant of cultures derived from CD28/CTLA4<sup>-/-</sup> mice ( $0.94 \pm 0.25$  ng/mL) and this amount was significantly less compared with cultures from OVA-sensitized and challenged wild-type mice stimulated with anti-CD3 mAb ( $7.97 \pm 1.45$  ng/mL,  $P < 0.001$ ). Antigenic re-stimulation of lung cell cultures from OVA-sensitized and challenged CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice did not result in cytokine production above medium control values.

So, these data suggest that T cells present in lung tissue derived from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice were not primed after sensitization and challenge with OVA *in vivo*, as determined by undetectable cytokine production upon OVA re-stimulation *ex vivo*. Interestingly, these unprimed T cells seemed to be characterized by an enhanced IFN-γ reactivity because these cultures produced significantly more IFN-γ upon polyclonal stimulation not only compared with wild-type mice sensitized and challenged with OVA but also in comparison with naïve wild-type mice (data not shown in Fig. 3).

## Discussion

A recent study using a novel mouse strain lacking both CD28 and CTLA4 demonstrated considerable residual CD4<sup>+</sup> T cell proliferation *in vitro* that was dependent on B7-1 or B7-2, suggesting the existence of an additional third B7-1/B7-2 receptor [13]. In addition, CD28/CTLA4<sup>-/-</sup> mice mounted a substantial Th1-mediated cardiac allograft rejection response *in vivo*, in contrast with long-term allograft survival in B7-1/B7-2<sup>-/-</sup> mice [13–15]. The role of this putative B7-1/B7-2 receptor in other T cell-mediated disease models is unknown at the moment. Therefore, in the present study, we investigated if T cell co-stimulation via the supposed B7-1/B7-2 receptor has a role in the induction of the Th2-dominated murine model of allergic asthma. Sensitization and aerosol challenge of wild-type mice with OVA resulted in airway hyper-responsiveness to methacholine and the influx of eosinophils in the BALF, cardinal airway features of patients suffering from allergic asthma. In addition, OVA-specific IgE in serum, IL-5 in the BALF and Th2 cytokine production by lung cells upon OVA re-stimulation *in vitro* were observed. In agreement with an earlier study [16], we

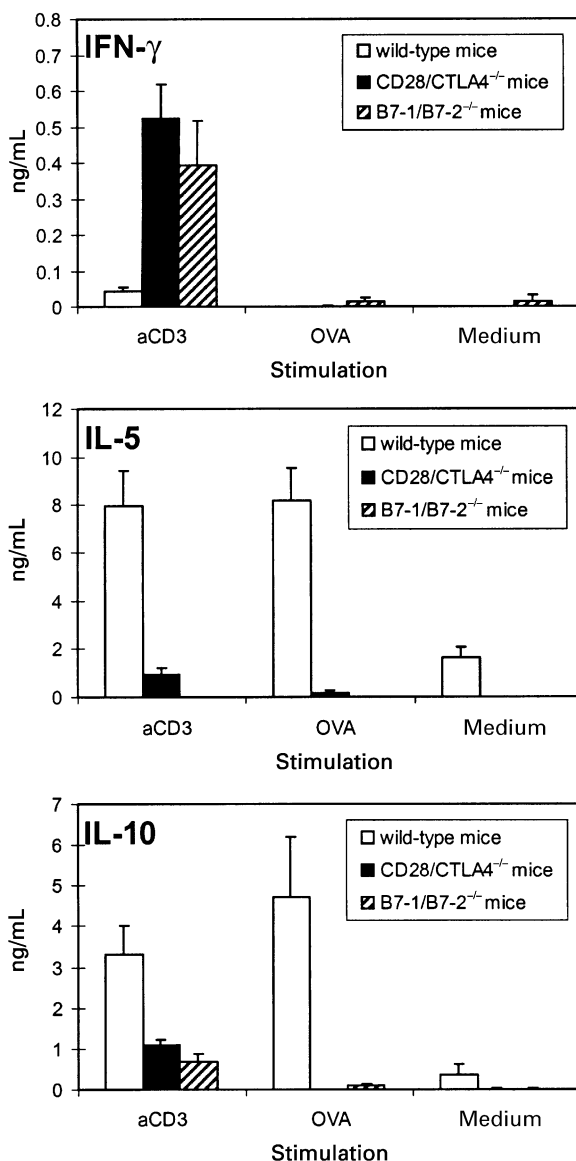


**Fig. 3.** Lung T cells from CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice are not primed. BALB/c wild-type (open bars), CD28/CTLA4<sup>-/-</sup> (closed bars) and B7-1/B7-2<sup>-/-</sup> mice (hatched bars) were sensitized to OVA adsorbed to alum and challenged with three OVA aerosols. At 24 h after the last OVA aerosol challenge, a single-cell suspension of lung tissue was prepared. Lung cells were stimulated for 5 days with 50 µg/mL anti-CD3 mAb or 10 µg/mL OVA or cultured with medium only and IFN-γ, IL-5 and IL-10 in the supernatant of the cultures was determined. Values are expressed as mean  $\pm$  SEM ( $n = 5$ –6 per group).

failed to induce any of the features of allergic airway sensitization in the B7-1/B7-2<sup>-/-</sup> mice. Interestingly, also CD28/CTLA4<sup>-/-</sup> mice showed no manifestations of allergic asthma upon OVA sensitization and challenge. So, these data clearly demonstrate that T cell co-stimulation *via* the putative B7-1/B7-2 receptor appears to have no role in the induction of Th2-mediated features of allergic airway sensitization in this murine model. Conversely, these data provide further evidence for the critical role of CD28 signalling in the development of Th2-dominated allergic inflammatory responses [17, 19, 23].

Previously, it has been demonstrated that no features of allergic airway sensitization can be induced in CD28<sup>-/-</sup> mice [17–19]. In these mice, however, the contribution of other molecules including the putative B7-1/B7-2 receptor to T cell co-stimulation might be masked by negative signalling *via* CTLA4 [20–22]. Following the observation that no manifestations of allergic asthma could be induced in CD28<sup>-/-</sup> mice, two research groups administered CTLA4-Ig to these mice [17, 19] to determine whether the absence of these manifestations was due exclusively to the lack of a positive signal by CD28 or whether an unopposed negative signal *via* CTLA4 was inhibiting the T cell response. Incongruent results were obtained because in one study CTLA4-Ig administration had no effect [17], whereas treatment of CD28<sup>-/-</sup> mice with CTLA4-Ig restored lymphocyte, but not eosinophil recruitment to the airways in the second study [19].

Importantly, CTLA4-Ig prevents the interaction of the B7-1 and B7-2 molecules with both CTLA4 and the putative B7-1/B7-2 receptor, thus not allowing for discrimination between these two pathways regulating T cell activation. In addition, treatment of mice with CTLA4-Ig might leave some B7-1 and B7-2 molecules uncovered [26] that may bind CTLA4 (and the supposed B7-1/B7-2 receptor), whereas deficiency of a gene encoding a particular molecule guarantees the complete absence of this molecule. Therefore, unravelling the potential role of co-stimulation by the putative B7-1/B7-2 receptor required the absence of both CD28 and CTLA4. This study demonstrates that the additional deficiency of the CTLA4 molecule in mice did not result in considerable residual manifestations of allergic asthma by unmasking co-stimulation *via* the supposed B7-1/B7-2 receptor.

In CD28<sup>-/-</sup> mice, the failure to induce manifestations of allergic asthma is accompanied by signs of residual T cell activation; lymphocytes and IFN- $\gamma$  are present in the BALF [17, 18] and lymphocytes obtained from the lungs, spleen and lymph nodes respond to antigenic re-stimulation *in vitro* [17–19]. Likewise, the absence of the key airway features in CD28/CTLA4<sup>-/-</sup> mice might leave open the possibility that OVA-specific CD4<sup>+</sup> T cells in these mice are in fact co-stimulated *via* the putative B7-1/B7-2 receptor, resulting in T cell activation that is not strong enough to induce airway pathology. However, the data presented in this report provide no evidence for residual T cell activation in CD28/CTLA4<sup>-/-</sup> mice. First, the amount of antigen-specific IgE in serum after OVA immunization was below the detection level, suggesting that no or insufficient systemic Th2 cells have developed because B cells require help by systemic Th2 cells for isotype switching to IgE [27]. It should be noted that airway hyper-responsiveness *in vivo* and airway eosinophilia can be induced

in mice in the absence of systemic IgE [28], but has been shown to be critically dependent on CD4<sup>+</sup> cells [29–32]. Second, T cells present in lung tissue derived from CD28/CTLA4<sup>-/-</sup> mice produced no cytokines upon antigenic re-stimulation *in vitro*, indicating that they were not primed upon OVA sensitization and challenge *in vivo*.

Yet it remained possible that OVA-specific CD28/CTLA4<sup>-/-</sup> CD4<sup>+</sup> cells in the lung draining lymph nodes were co-stimulated *via* the putative B7-1/B7-2 receptor, but had failed to migrate to the airways. To test this hypothesis, also the thoracic lymph nodes (TLNs) were isolated at 24 h after the last OVA aerosol challenge in the presented experiment. Whereas wild-type, CD4<sup>+</sup> lymphocytes produced a small amount of IFN- $\gamma$  and fair amounts of IL-4, IL-5 and IL-10 upon polyclonal stimulation *in vitro*, no cytokines were detectable in the supernatant of CD4<sup>+</sup> TLN cell cultures derived from both CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice (data not shown). In addition, the percentage of CD4<sup>+</sup> cells in the TLN was very similar in OVA-sensitized and challenged CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2 mice, indicating that no residual CD4<sup>+</sup> T cell expansion had occurred in CD28/CTLA4<sup>-/-</sup> mice. Finally, FACS analysis demonstrated no enhanced expression of activation markers such as CD25, CD62L and CD69 on CD4<sup>+</sup> TLN cells obtained from CD28/CTLA4<sup>-/-</sup> mice in comparison with B7-1/B7-2<sup>-/-</sup> mice (data not shown). In conclusion, we were unable to demonstrate any sign of residual T cell activation in both the TLNs and lungs from CD28/CTLA4<sup>-/-</sup> mice upon OVA-sensitization and challenge *in vivo*. However, this study cannot exclude the possibility that after initial T cell activation in the presence of CD28, the supposed B7-1/B7-2 receptor is involved in a later phase of OVA-specific T cell activation.

The discrepancy of residual T cell activation in CD28<sup>-/-</sup> mice [17–19] vs. no signs of T cell activation in CD28/CTLA4<sup>-/-</sup> mice in this experiment might be explained by the different antigens and protocols used for the induction of manifestations of allergic asthma. For example, Mathur et al. [17] used eggs and antigen of the *Schistosoma mansoni* parasite and Lambrecht et al. [18] passively sensitized naïve CD28<sup>-/-</sup> mice with OVA-pulsed myeloid dendritic cells.

Antigenic re-stimulation of lung cell cultures from OVA-sensitized and challenged CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice did not result in Th1 or Th2 cytokine production above medium control values. Interestingly, the seemingly unprimed T cells from both OVA-sensitized and challenged CD28/CTLA4<sup>-/-</sup> and B7-1/B7-2<sup>-/-</sup> mice were characterized by an enhanced IFN- $\gamma$  reactivity. Lung cell cultures derived from these mice produced more IFN- $\gamma$  upon polyclonal stimulation, not only compared with wild-type mice displaying Th2-mediated manifestations of asthma upon OVA sensitization and challenge but also compared with naïve wild-type mice. In agreement herewith, BALF CD4<sup>+</sup> T cells obtained after challenge from passively sensitized CD28<sup>-/-</sup> mice were shown to have an increased tendency to produce IFN- $\gamma$  upon polyclonal stimulation compared with wild-type mice [18]. Moreover, CD4<sup>+</sup> CD28/CTLA4<sup>-/-</sup> cells produced more IFN- $\gamma$  and less IL-4 and IL-10 upon secondary polyclonal stimulation *in vitro* compared with wild-type CD4<sup>+</sup> cells [13]. These findings are consistent with the importance of CD28 for Th2 differentiation [23]. In addition, this observation might explain why it seems impossible to

prime antigen-specific T cells towards the Th2 phenotype – as opposed to a Th1-mediated cardiac allograft rejection [13, 15, 22, 33] – even though a protocol was used that normally results in very strong Th2-mediated inflammatory responses in BALB/c mice. However, the enhanced IFN- $\gamma$  reactivity did not prime OVA-specific T cells towards the Th1 phenotype either as observed locally in lung T cell re-stimulation and systemically by the absence of the Th1 cell-dependent IgG2a isotype [34]. In wild-type mice, besides a high level of IgE also OVA-specific IgG2a can be detected upon sensitization and challenge with OVA (data not shown).

With the caution that a single strain of mice and a single protocol for the induction of features of allergic airway sensitization has been used, the putative B7-1/B7-2 receptor apparently has a differential role in Th1-mediated cardiac allograft rejection and Th2-mediated allergic asthma. Perhaps, the expression of the supposed B7-1/B7-2 receptor is differentially regulated on Th1 vs. Th2 cells. Indeed, T helper cells can be divided not only on the basis of their cytokine profile but also on their differential expression (kinetics) of cell surface molecules such as chemokine receptors and the CD28-related inducible T cell co-stimulator (ICOS) [35–37]. Future studies regarding the induction of other T cell-mediated diseases in CD28/CTLA4<sup>-/-</sup> mice might help to clarify the precise role of the supposed B7-1/B7-2 receptor in T cell responses. Ultimately, cloning of the gene encoding the putative B7-1/B7-2 receptor will greatly facilitate research on this receptor.

Whereas CD28-deficient mice are still capable of raising a number of *in vivo* immune responses, including cardiac allograft rejection [13, 15, 22, 33], induction of allogeneic graft-versus-host disease [38], generation of cytotoxic T cells [39] and certain delayed-type hypersensitivity responses [40], the present study provides further evidence for the critical role of CD28 signalling in the development of Th2-dominated allergic inflammatory responses [17–19, 23]. Moreover, as anticipated by the data on the failure to induce features of allergic airway sensitization in B7-1/B7-2<sup>-/-</sup> mice (Ref. [16] and this study), T cell co-stimulatory receptors that interact with ligands other than B7-1 and B7-2, e.g. ICOS, appear to play no major role in the induction of asthma manifestations in this model. So, blockade of CD28 in allergic asthma appears to be a very promising therapeutic option.

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