

## ORIGINAL ARTICLE

# Antigen dose-dependent suppression of murine IgE responses is mediated by CD4<sup>−</sup>CD8<sup>−</sup> double-negative T cells

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## Clinical & Experimental Allergy

### Summary

**Background** The IgE response against protein antigens is profoundly influenced by the dose used for sensitization.

**Objective** The aim of the study was to identify immune cells that are involved in antigen dose-dependent regulation of IgE formation.

**Methods** Wild-type mice as well as T helper (Th)1-deficient IL-12p40<sup>−/−</sup> and IFN-γ<sup>−/−</sup> mice were immunized by repeated intraperitoneal injection of either low doses (K01 mice) or high doses (K100 mice) of keyhole limpet haemocyanin adsorbed to aluminium hydroxide. Splenocytes of immunized mice were restimulated *in vitro* and antigen-dependent T cell proliferation and cytokine production were measured. The frequency of regulatory T cell subsets among splenocytes from K01 and K100 mice was compared using fluorocytometry and RT-PCR analysis. Splenocytes or T cell subpopulations were transferred into naïve mice and the effect of lymphocyte transfer on IgE production after priming of recipients with low antigen doses was determined.

**Results** Specific IgE production was considerably impaired in K100 mice. Antigenic restimulation revealed hypoproliferation of K100 splenocytes and reduced production of Th2 cytokines IL-4, IL-5 and IL-13, but no induction of IFN-γ production. Moreover, lymphocytes from K01 and K100 mice did not show significant differences in the expression of molecules associated with the phenotype or activity of conventional regulatory T cells. Transfer of splenocytes or purified T cells from K100 mice substantially suppressed the induction of IgE production in the recipients in an antigen- and isotype-specific manner. Neither CD4<sup>+</sup> nor CD8<sup>+</sup> T cells from K100 mice were able to inhibit IgE formation; instead, we identified CD4<sup>−</sup>CD8<sup>−</sup> double-negative T cells (dnT cells) as the principal T cell population, which potentially suppressed IgE production.

**Conclusion** Our data demonstrate that CD4<sup>−</sup>CD8<sup>−</sup> dnT cells play a major role in the regulation of IgE responses induced by high antigen doses.

**Keywords** allergy, antigen dose, CD4<sup>−</sup>CD8<sup>−</sup> double-negative T cells, immune tolerance, IgE, keyhole limpet haemocyanin, mouse model, regulatory T cells

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### Introduction

The prevalence of type I hypersensitivities such as allergic bronchial asthma or allergic rhino-conjunctivitis has increased dramatically over the past decades [1]. It is well accepted that the central event in the pathogenesis of these atopic disorders is the excessive and sustained secretion of antibodies of the IgE class by allergen-reactive B cells. A critical parameter for the induction or inhibition of IgE responses is the allergen dose. The clinical benefit from specific immunotherapy (SIT), which

is based upon subcutaneous injection or sublingual application of rapidly increasing doses of standardized allergen extracts, has been clearly documented to be related to the application of high allergen doses at the maintenance phase [2, 3]. The therapeutic efficacy of SIT is correlated with the induction of a state of tolerance to natural allergen exposure, comprising the induction of IL-10- or TGF-β-producing regulatory T cells (Tregs), which antagonize the dominance of IgE-promoting allergen-specific T helper (Th)2 cells in allergic individuals [4]. Consequently, allergen-specific IgE in serum gradually decreases over a

period of months or years of SIT [5]. Similarly, the balance between allergen-specific IL-10- or TGF- $\beta$ -producing Tregs and Th2 cells appears to be decisive for the development of healthy or allergic immune responses upon a natural encounter with allergens [6, 7].

The antigen dose likewise represents a critical experimental variable to induce adequate sensitization in mouse models of type I hypersensitivities. Immunization of mice with low doses of antigen elicits considerable IgE production, whereas immunization with high doses of antigen results in the suppression of IgE but not IgG formation [8–10]. The antigen dose-dependent regulatory mechanism inhibiting the differentiation of B cells into IgE-producing plasma cells *in vivo* is yet unknown. In this report, we focus on the involvement of T lymphocytes with a regulatory function in B cell differentiation following immunization with high antigen doses and analyse a possible role for Tregs in the suppression of IgE production. We have identified CD4<sup>+</sup>CD8<sup>+</sup> double-negative T cells (dnT cells) as a suppressor cell population inhibiting the generation of IgE responses *in vivo*.

## Material and methods

### *Mice and immunization protocol*

BALB/c, C57BL/6, IL-12p40<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice were bred and maintained in the Central Animal Facilities of the University of Mainz in specific pathogen-free conditions on a standard diet. The 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985) were followed. The experiments were approved by the Ethics Commission according to the German Law on the Protection of Animals. Animals were 6–8 weeks old at the beginning of the experiments. Mice were immunized intraperitoneally in intervals of 2 weeks with low doses (0.1  $\mu$ g/injection; K01 mice) or high doses (100  $\mu$ g/injection; K100 mice) of keyhole limpet haemocyanin (KLH; Sigma-Aldrich, Deisenhofen, Germany) or with the indicated doses of ovalbumin (OVA; Calbiochem, La Jolla, CA, USA), dissolved in 100  $\mu$ L phosphate-buffered saline (PBS) and adsorbed to an equal volume of Imject<sup>®</sup> Alum (Pierce, Rockford, IL, USA) as an adjuvant containing 4 mg aluminium hydroxide.

### *Determination of antibodies in serum*

At the indicated timepoints, immunized mice were bled by puncture of the retro-orbital plexus. Sera were collected and kept frozen at  $-20^{\circ}\text{C}$  until thawed for the determination of KLH- or OVA-specific Ig subclasses by antigen capture ELISA as reported [11, 12]. The antibody titre was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis. In transfer experiments, IgE content was normal-

ized to the mean titre of the control group (without transfer) of the respective experiment.

### *RBL-2H3 degranulation assay*

IgE-mediated immediate-type reactions were determined *in vitro* using the rat basophil leukaemia cell line RBL-2H3 as reported [13] with the modification that KLH (1  $\mu$ g/mL) was used as the antigen to trigger IgE-dependent degranulation of RBL-2H3 cells and the release of  $\beta$ -hexosaminidase. The amount of  $\beta$ -hexosaminidase released into the medium is expressed as the enzymatic activity determined in the culture supernatant in relation to the total enzymatic activity (sum of the enzymatic activities in the supernatant and in the corresponding RBL-2H3 cell lysate).

### *Proliferation assay*

To reveal the presence of suppressor cells in spleens of K01 and K100 mice, splenocytes were cultured at a cell density of  $5 \times 10^5$  and  $5 \times 10^4$  cells/well, respectively, on 96-well flat-bottomed microtitre plates (Corning Costar, Bodenheim, Germany) in a volume of 200  $\mu$ L culture medium [Iscove's Modified Dulbecco's Medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; PAN Systems GmbH, Nürnberg, Germany), 50  $\mu$ M 2-mercaptoethanol (Carl Roth GmbH&Co), 2 mM L-glutamine and 100 U/mL penicillin/100  $\mu$ g/mL streptomycin (Gibco)] alone or with various concentrations of KLH. After 3 days, cells were incubated with  $^3\text{H}$ -thymidine (1  $\mu$ Ci/well) for another 16 h. Levels of incorporated  $^3\text{H}$ -thymidine were determined by liquid scintillation counting.

### *Determination of cytokines in culture supernatants*

Splenocytes of K01 and K100 mice were incubated for 72 h on 24-well tissue culture plates ( $5 \times 10^6$  cells/well) (Corning Costar) in a volume of 1 mL culture medium with or without KLH (1  $\mu$ g/mL). After 3 days, cytokine (IL-4, IL-5, IL-10, IL-13, IFN- $\gamma$ , TGF- $\beta$ 1) contents in culture supernatants were quantified by sandwich-ELISA as described previously [12, 14].

### *Flow cytometry*

Splenocytes of K01 and K100 mice were washed in staining buffer (PBS/2% FCS). Cells ( $2\text{--}5 \times 10^5$ ) were incubated for 15 min at room temperature with the monoclonal antibody 2.4.G2 [15] against CD16/32 (Fc  $\gamma$  RII/III) to block Fc receptor-mediated unspecific staining. Subsequently cells were incubated (20 min, on ice) with primary antibodies as indicated. Fluorescein isothiocyanate-conjugated antibodies recognized CD152/cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (clone 63828) and

glucocorticoid-induced TNFR-related protein (GITR) (clone 108619) (both R&D Systems, Minneapolis, MN, USA), phycoerythrin (PE)-labelled antibodies recognized CD25 (clone PC61 5.3; Caltag, Burlingame, CA, USA) and phycoerythrin cyanin 5-labelled antibodies recognized CD4 (clone RM4-5) and CD8a (clone 53-6.7) (both BD Biosciences, San Diego, CA, USA). Biotinylated antibodies recognized CD28 (clone 37.51.1; Caltag) and  $\gamma\delta$  T cell receptor (clone GL3; BD Biosciences), and were detected using 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein-conjugated streptavidin. Suitable isotype controls were used. For intracellular staining of transcription factor forkhead box P3 (Foxp3), cells were subsequently treated with the Foxp3 staining buffer set (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions in order to achieve permeabilization. Subsequently, the cells were stained with PE-conjugated antibodies recognizing Foxp3 (clone 3G3; Miltenyi Biotec). Flow cytometric analysis was performed using a FACScan flow cytometer (BD Biosciences) equipped with CellQuest Software. Lymphocytes were gated due to size (FSC) and granularity (SSC) and at least 10 000 events in this gate were acquired for the analysis of surface marker expression.

#### *Real-time polymerase chain reaction analysis*

To assess transcriptional changes of markers of Tregs (Foxp3, IL-10, TGF- $\beta$ 1) at the mRNA level, splenocytes ( $5 \times 10^6$  cells/well) of K01 and K100 mice were restimulated with 1  $\mu$ g/mL KLH *in vitro* on 24-well tissue culture plates. After 72 h, cells were collected and total RNA was isolated using the NucleoSpin RNAII Total RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to the protocol recommended by the manufacturer. RT-PCR was performed as described before [16], using primer sequences published previously [12].

#### *Magnetic cell separation*

Splenocytes of K01 and K100 mice were prepared and CD4<sup>+</sup>, CD8<sup>+</sup> and CD90<sup>+</sup> T cells were positively selected by magnetic separation using the MACS technology (Miltenyi Biotec). For this, splenocytes were resuspended in ice-cold separation buffer (PBS with 2 mM ethylenediaminetetraacetic acid and 2% FCS) and incubated with anti-CD4, anti-CD8 and anti-CD90 MicroBeads, respectively, as recommended by the manufacturer. The cell suspension was passed over a separation column (Type MS) using the MiniMACS separator system, and magnetically labelled cells were eluted from the column using a plunger according to the manufacturer's instructions.

For negative selection of untouched T cells using the PAN T cell Isolation Kit, splenocytes, resuspended in separation buffer, were first incubated with a biotin-conjugated antibody cocktail labelling non-T cells, which

are erythrocytes, B cells, dendritic cells, macrophages, granulocytes and NK cells as well as NKT cells. Subsequently biotinylated antibodies were labelled with anti-biotin MicroBeads according to the manufacturer's instructions. After passing over a separation column (LS type) using the MidiMACS separator system, the effluent was collected as untouched T cell fraction.

CD4<sup>+</sup>CD8<sup>+</sup> dnT cells were isolated from splenocytes by a combined magnetic cell separation strategy. First, untouched T cells were enriched using the PAN T cell Isolation Kit as described. Then, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were depleted from the T cell fraction by positive selection as described using a mixture of anti-CD4 and anti-CD8 MicroBeads. The CD4<sup>+</sup>CD8<sup>+</sup> dnT cells were highly purified in the effluent passing through the column.

The purity of the different T cell populations was verified by cytofluorometric analysis of gated lymphocytes and constituted  $92.4 \pm 1.8\%$  ( $n = 5$ ) and  $95.7 \pm 1.8\%$  ( $n = 3$ ) CD3<sup>+</sup> T cells for positively and negatively selected total T cells, respectively,  $94.5 \pm 1.3\%$  ( $n = 3$ ) for CD4<sup>+</sup> T cells,  $93.3 \pm 0.7\%$  ( $n = 3$ ) for CD8<sup>+</sup> T cells and  $89.3 \pm 5.3\%$  ( $n = 2$ ) for CD4<sup>+</sup>CD8<sup>+</sup> T cells.

#### *Adoptive transfer of lymphocytes*

Unseparated splenocytes ( $5 \times 10^6$ ) or isolated T cell subpopulations ( $2-5 \times 10^6$  cells), respectively, were injected intravenously into naïve syngeneic mice. After 24 h, recipients and control mice, which had not received donor cells, were sensitized with low doses of KLH as described above.

#### *Statistical analysis of data*

Data are presented as mean  $\pm$  SEM. Statistical evaluation of the experimental data was performed by non-parametric Wilcoxon's rank-sum test using 'SigmaStat' software. A value of  $P < 0.05$  was considered statistically significant.

### **Results**

#### *Immunization with low antigen doses promotes immunoglobulin E production, whereas immunization with high antigen doses suppresses immunoglobulin E responses*

Repeated intraperitoneal injection of BALB/c mice in intervals of 14 days with 0.1  $\mu$ g KLH per injection adsorbed to Alum as an adjuvant (K01 mice) induced vigorous production of specific IgE antibodies during the course of five immunizations (Fig. 1a). In contrast, K100 mice, immunized five times with 100  $\mu$ g KLH per injection plus adjuvant, showed an almost complete lack of IgE antibodies in their sera. The virtual absence of IgE production in K100 mice was verified on a functional

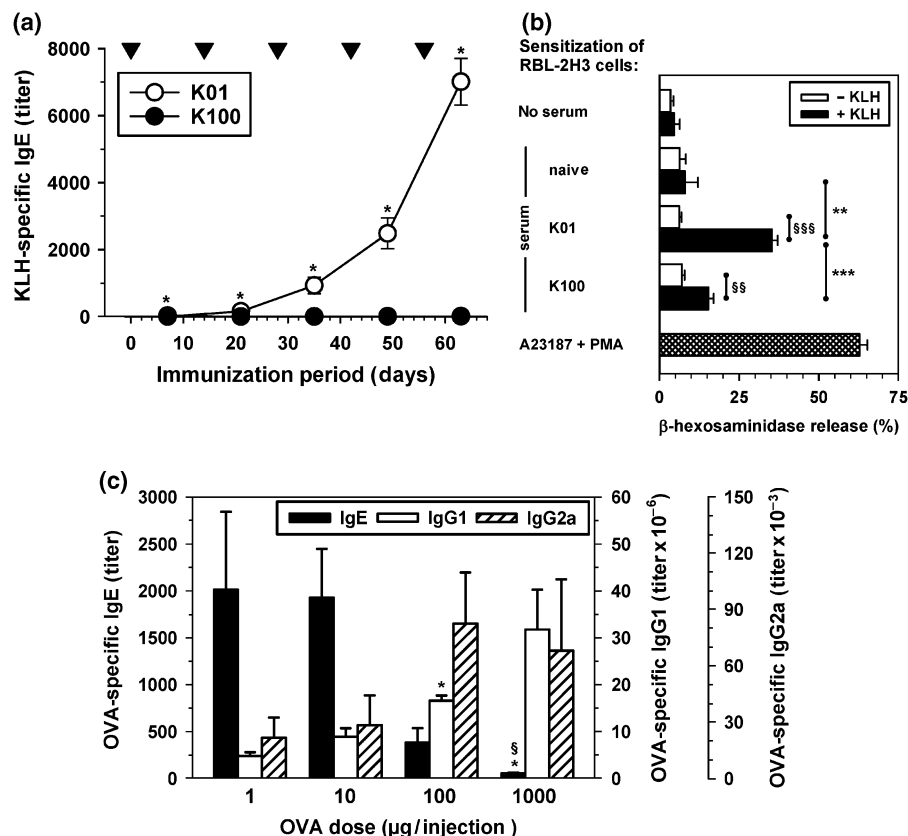


Fig. 1. Divergent IgE production after immunization with low or high antigen doses. (a) Sera of BALB/c mice immunized in intervals of 14 days (black triangles) by an intraperitoneal injection of 0.1  $\mu$ g keyhole limpet haemocyanin (KLH) (K01; open circles) or 100  $\mu$ g KLH (K100; closed circles) adsorbed to Alum were taken 7 days after each immunization and levels of KLH-specific IgE were determined by ELISA. Data are presented as mean titres  $\pm$  SEM of individual mice ( $n = 4$ /group). \* $P < 0.05$ , vs. K100 mice. Graphs are representative of five independent experiments. (b) Anaphylactic activity of BALB/c K01 and K100 sera, recovered 7–10 days after the fifth immunization with KLH, was determined *in vitro*. After incubation of rat basophil leukaemia (RBL)-2H3 cells with sera, IgE-dependent activation and  $\beta$ -hexosaminidase release were induced with KLH (closed bars) and compared with degranulation of cells cultured without KLH (open bars). Data are presented as mean release  $\pm$  SEM of 16 individual sera obtained in three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , vs. K01 sera; §§ $P < 0.01$ , §§§ $P < 0.001$ , without vs. with KLH. (c) Sera of BALB/c mice were recovered 7 days after the fifth immunization with the indicated doses of ovalbumin (OVA) adsorbed to Alum and levels of OVA-specific IgE, IgG1 and IgG2a were determined by ELISA. Data are presented as mean titres  $\pm$  SEM of individual mice ( $n = 4$ /group). \* $P < 0.05$  and § $P < 0.05$ , vs. mice immunized with 1 and 10  $\mu$ g OVA, respectively.

level by the determination of the IgE-dependent anaphylactic potential of sera from immunized mice in a RBL-2H3 basophil degranulation assay *in vitro*. Sera recovered from K01 mice exhibited high anaphylactic activity and triggered substantial antigen-dependent release of  $\beta$ -hexosaminidase from RBL-2H3 cells (Fig. 1b), which constituted approximately 56% of the maximum release induced by IgE-independent activation of RBL-2H3 cells with calcium ionophor A23187 and phorbol-12-myristate-13-acetate. In comparison, sera from K100 mice showed a strongly decreased capacity to provoke the degranulation of RBL-2H3 cells, which was marginally higher than but did not significantly differ ( $P = 0.107$ ) from degranulation originating from incubation of RBL-2H3 cells with sera from naïve BALB/c mice, representing the background release of  $\beta$ -hexosaminidase in this assay.

In addition, we demonstrated antigen dose dependency of the IgE response to OVA used as a model allergen, being

high after five immunizations with low doses of 1 or 10  $\mu$ g OVA per injection, but marginal after five immunizations with high doses such as 1000  $\mu$ g OVA (Fig. 1c). In contrast to IgE, antigen-specific IgG1 as well as IgG2a titres were elevated in sera of mice immunized with high doses of OVA, confirming our former findings with KLH showing that the failure of observing IgE immune responses in mice immunized with high antigen doses is isotype-specific and not due to a general deficiency in B cell activation [10].

#### Suppression of immunoglobulin E production after immunization with high antigen doses is not due to the induction of T helper 1 cells

To exclude that a shift towards the differentiation of Th1 cells plays a major role in the inhibition of IgE formation in K100 mice, we investigated whether C57BL/6 IFN- $\gamma^{-/-}$  mice or C57BL/6 IL-12p40 $^{-/-}$  mice, which are defective in

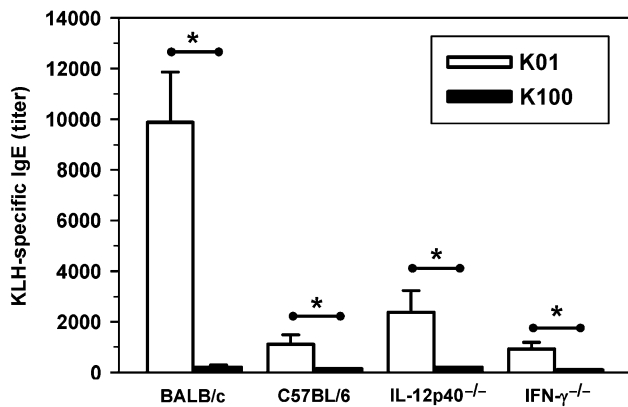


Fig. 2. Decreased IgE production in wild-type as well as Th1-deficient mice immunized with high antigen doses. Sera of wild-type and Th1-deficient mice were recovered 7 days after the fifth immunization (K01, open bars; K100, filled bars) and levels of keyhole limpet haemocyanin (KLH)-specific IgE in sera were determined by ELISA. Data are presented as mean titres  $\pm$  SEM of individual mice ( $n = 4$ /group). \* $P < 0.05$ , K01 vs. K100 mice.

the generation of Th1 immune responses [17, 18], showed similar characteristics of antigen dose-dependent regulation of IgE production. Unlike BALB/c mice, which are known as so-called 'high-responder' mice for IgE synthesis [19], C57BL/6 mice are rather considered as 'intermediate-responder' mice due to variations in the genetic background [19]. In line with this view, wild-type C57BL/6 K01 mice developed substantial IgE titres after five injections of KLH, which are, however, significantly lower than those in BALB/c K01 mice ( $P < 0.05$ ) (Fig. 2). In accordance with the situation in BALB/c mice, priming with high doses of KLH efficiently prevented IgE production in C57BL/6 K100 mice as well. Likewise, IgE immune responses were significantly suppressed in Th1-deficient C57BL/6 IFN- $\gamma$ <sup>-/-</sup> K100 or C57BL/6 IL-12p40<sup>-/-</sup> K100 mice as compared with K01 mice from the same genetic background (Fig. 2). This finding implicates that Th1 cells are not involved in the inhibition of IgE production after immunization with high antigen doses.

#### *Splenocytes from mice, immunized with high antigen doses, are hyporeactive*

We next assessed antigen-specific T cell reactivity in KLH-primed mice by culturing splenocytes from BALB/c K01 and K100 mice, respectively, for 3 days in the presence of various concentrations of KLH. *In vitro* stimulation with exogenous protein preferentially leads to MHC class II-restricted antigen presentation, and thus induces proliferation of and cytokine release mainly by CD4<sup>+</sup> Th cells. Splenocytes from both K01 and K100 mice, cultured at a low cell density of  $5 \times 10^4$  cells/well, proliferated efficiently after restimulation with increasing amounts of KLH (Fig. 3a). Hence, the absolute proliferation determined at the highest concentration of KLH tested

(30  $\mu$ g KLH/mL) in several independent experiments did not significantly differ in cultures of K01 and K100 spleen cells (K01  $48\,102 \pm 4733$  c.p.m.; K100  $39\,323 \pm 4228$  c.p.m.;  $P = 0.204$ ;  $n = 5$  experiments). When splenocytes from K01 mice were restimulated at high cell numbers ( $5 \times 10^5$  cells/well), they also proliferated efficiently (Fig. 3b). The shift in the antigen dose response curve towards lower concentrations of KLH is in accordance with earlier studies [20]. In contrast, proliferation of splenocytes from K100 mice cultured under high density conditions was significantly lower (Fig. 3b) (proliferation at 30  $\mu$ g KLH/mL: K01  $44\,909 \pm 3249$  c.p.m.; K100  $15\,483 \pm 2316$  c.p.m.;  $P = 0.0018$ ;  $n = 5$  experiments). The reduced reactivity of K100 T cells was also evident when cytokine production by the stimulated splenocytes was assessed. Whereas K01 T cells released substantial amounts of the Th2 cytokines IL-4, IL-5 and IL-13 into the culture supernatant, secretion of these cytokines by K100 T cells was largely impaired (Fig. 3c). Moreover, in cultures of both groups, no antigen-specific induction of IFN- $\gamma$  production was noticed (Fig. 3c). The impairment of T cell responsiveness under high-density culture conditions opens up the possibility that regulatory T cells exist among K100 splenocytes, which are present at a low frequency and/or exert their suppressive effect in a cell contact-dependent manner.

#### *The frequency of conventional Tregs is not increased after immunization with high antigen doses*

We investigated by FACS analysis whether the incidence of conventional Treg subpopulations was augmented among K100 splenocytes. As shown in Fig. 4a, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells, considered as nTregs, among lymphocytes was not significantly different in K01 and K100 mice, irrespective of whether these cells coexpressed additional molecules that are associated with the function or the phenotype of nTregs such as CTLA-4 [21] or GITR [22]. Likewise, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells among the lymphocyte population of splenocytes of K01 and K100 mice that were positive for the expression of the transcription factor Foxp3, which is characteristic for nTregs as well as the majority of adaptive Treg populations [23, 24], was comparable. Moreover, the occurrence of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> lymphocytes among mediastinal (K01:  $2.94 \pm 0.51\%$ ; K100:  $2.11 \pm 0.25\%$ ;  $P = 0.400$ ,  $n = 3$  experiments) or mesenteric (K01:  $3.78 \pm 0.36\%$ ; K100:  $3.37 \pm 0.51\%$ ;  $P = 0.552$ ,  $n = 3$  experiments) lymph node cells did not significantly differ in K01 and K100 mice. In accordance with the FACS data, mRNA expression of Foxp3 was not significantly enhanced ( $P = 0.223$ ;  $n = 6$  experiments) in K100 splenocytes as determined by quantitative RT-PCR analysis (Fig. 4b). Referring to adaptive Treg populations, we concluded from the quantitative RT-PCR analysis of mRNA expression that formation of the immunoregulatory cytokine TGF- $\beta$ 1 by K01 and K100 splenocytes was not different (Fig. 4b). This supposition was

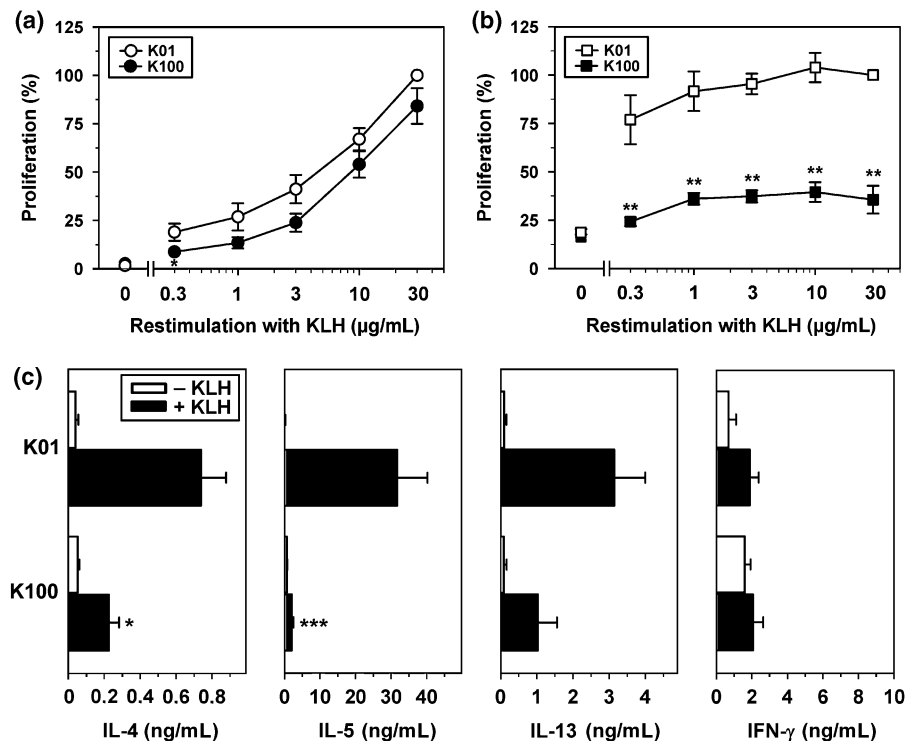


Fig. 3. T cell activation is decreased after immunization with high antigen doses. BALB/c mice were immunized five times as described in the legend to Fig. 1. (a, b) Seven to 10 days after the fifth immunization splenocytes of BALB/c K01 mice (open symbols) or K100 mice (filled symbols) were prepared and cultured in triplicates at a cell density of  $5 \times 10^4$  cells/0.2 mL/well (a) or  $5 \times 10^5$  cells/0.2 mL/well (b) for 96 h without or with the indicated concentrations of keyhole limpet haemocyanin (KLH). Proliferation was determined by the incorporation of  $^3\text{H}$ -thymidine (1  $\mu\text{Ci}$ /well) during the last 16 h of the culture period. Data are expressed as percentage of the proliferation value obtained for the stimulation of K01 splenocytes at the respective cell density with 30  $\mu\text{g}$  KLH/mL (100%). Results are presented as mean relative proliferation  $\pm$  SEM of splenocytes from individual mice ( $n = 5$ ). (c) Splenocytes ( $5 \times 10^6$  cells/mL/well) of BALB/c K01 and K100 mice, respectively, were stimulated for 72 h in triplicates without (open bars) or with (closed bars) 1  $\mu\text{g}$ /mL KLH. The indicated cytokines in culture supernatants were quantitated by ELISA. Data are presented as mean cytokine content  $\pm$  SEM in splenocyte cultures of individual mice ( $n = 4-7$ ). \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ , vs. K01 splenocytes.

confirmed by the determination of the content of TGF- $\beta$ 1 protein in supernatants of K01 and K100 splenocyte cultures (Fig. 4c). Additionally, mRNA expression of IL-10, another immunosuppressive cytokine produced by adaptive Tregs, was significantly lower in K100 splenocytes (Fig. 4b), which corresponds with ELISA data indicating that antigen-specific secretion of IL-10 protein by K100 splenocytes was significantly reduced (Fig. 4c). Furthermore, other T cell populations with suppressor cell function such as  $\gamma\delta$  T cells [25, 26],  $\text{CD}8^+$  T cells [27, 28] or  $\text{CD}8^+\text{CD}28^-$  T cells [29] also were not more prevalent among K100 splenocytes (Fig. 4a). In summary, our data suggest that the frequency of conventional Tregs in mice immunized with high antigen doses was not increased.

#### *Suppression of immunoglobulin E production, induced by immunization with high antigen doses, is mediated by $\text{CD}4^-\text{CD}8^-$ double-negative T cells*

If T cells with regulatory properties for IgE production were effective in K100 mice, the suppressive activity of these cells should be transferable to naïve mice. Therefore,

splenocytes from K01 mice and K100 mice, respectively, were transferred by intravenous injection into naïve recipients, which were subsequently immunized five times with low doses of KLH and were then analysed for IgE production. Control mice without adoptive transfer showed considerable KLH-specific IgE titres in the sera (mean IgE titre:  $3263 \pm 617$ ;  $n = 38$  mice) (Figs 5 and 6). The IgE levels in the sera of recipients of K100 splenocytes moderately rose after the first two injections of KLH (Fig. 5a). This might probably reflect a temporary activation of KLH-specific B memory cells among K100 splenocytes, which after transfer were no longer subject to the immediate negative control of the putative suppressor cells as they had been before in the microenvironment within the lymphatic organs of K100 mice, and which therefore are supposed to be able to develop into IgE-secreting plasma cells *in situ* [30]. However, the increase in IgE production was only transient, as in contrast to control mice without transfer that developed high IgE titres, the IgE titres in the recipients persisted on a low level in spite of further applications of low doses of KLH (Figs 5a and b), indicating that suppressor cells had been transferred

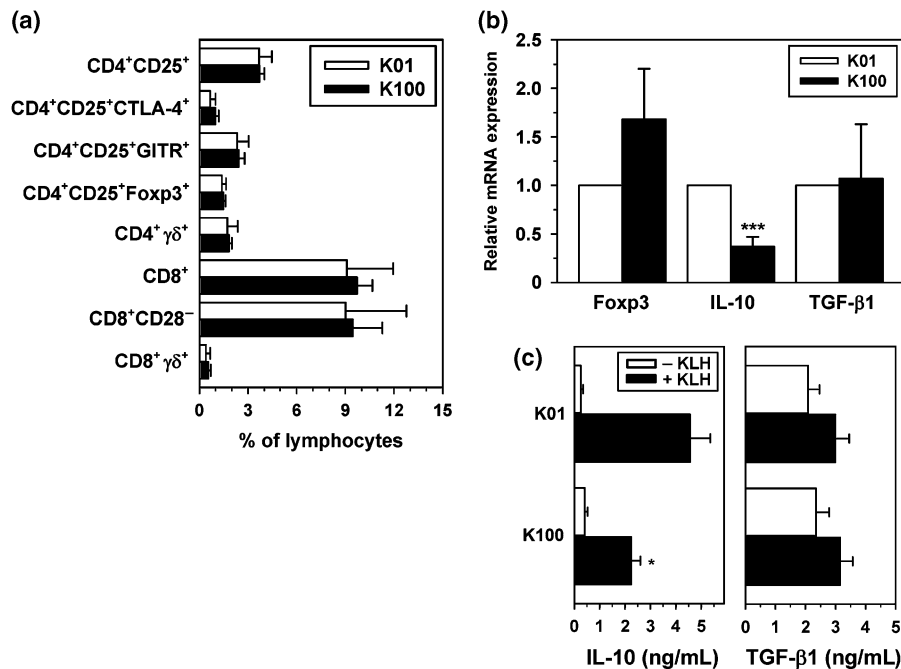


Fig. 4. Expression of marker molecules of conventional Tregs or immunomodulatory cytokines is not enhanced after immunization with high antigen doses. BALB/c mice were immunized five times as described in the legend to Fig. 1 and splenocytes were prepared from K01 mice (open bars) or K100 mice (filled bars) 7–10 days after the fifth immunization. (a) Splenocytes of BALB/c were labelled with fluorochrome-conjugated monoclonal antibodies against the indicated surface molecules or transcription factor forkhead box P3 (Foxp3) and analysed by fluorocytometry. Data are presented as mean percentage of positive cells  $\pm$  SEM in the lymphocyte population of individual mice ( $n = 4-10$ ). (b, c) Splenocytes ( $5 \times 10^6$  cells/well) were stimulated for 72 h with 1  $\mu$ g/mL keyhole limpet haemocyanin (KLH). (b) RNA was isolated and mRNA expression was analysed by quantitative RT-PCR. Data are presented as mean expression  $\pm$  SEM in splenocyte cultures of individual mice ( $n = 5-6$ ). (c) Levels of IL-10 and TGF-β1 in culture supernatants were quantitated by ELISA. Data are presented as mean cytokine content  $\pm$  SEM in splenocyte cultures of individual mice ( $n = 6-7$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$ , vs. K01 splenocytes.

within the K100 splenocyte population. Moreover, transfer of splenocytes from K01 mice did not affect the development of the IgE response in the recipients (Fig. 5b). In contrast to IgE production, IgG production was only marginally reduced by the transfer of K100 as well as K01 splenocytes (Fig. 5b). To analyse whether the inhibitory effect of transfer of K100 splenocytes on IgE production was antigen-specific, recipients were repeatedly immunized either with low doses of KLH or with the irrelevant antigen OVA used at a dose that induces high IgE titres (see Fig. 1). Whereas the KLH-specific IgE response was significantly decreased after transfer of splenocytes from K100 mice, IgE production in OVA-immunized recipients was similar to the one in OVA-primed control mice without transfer (Fig. 5c). These results indicate that the suppressor cells act in an antigen-specific manner.

Next, we determined in a series of experiments, whether the inhibitory activity of K100 splenocytes can be attributed to T cells or specific T cell subpopulations. Therefore, total T cells were isolated from K100 mice by direct magnetic labelling (positive selection of CD90<sup>+</sup> T cells) or by a negative selection strategy using an indirect magnetic labelling system (PAN T), which led to the depletion of non-T cells, namely B cells, dendritic cells, macro-

phages, granulocytes, NK cells as well as NKT cells. To standardize the effect of T cell transfer on IgE production, IgE titres in all test sera of an individual experiment were normalized to the mean titre of the respective control group without transfer. Transfer of T cells originating from K100 mice significantly inhibited the establishment of a profound IgE response in the recipient mice, whereas CD90<sup>+</sup> T cells purified from K01 mice did not significantly reduce IgE titres following adoptive transfer (Fig. 6). In order to characterize the suppressor T cells more closely, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells, respectively, were positively selected by direct magnetic labelling and transferred separately into naïve mice. Actually, neither CD4<sup>+</sup> nor CD8<sup>+</sup> T cells modulated IgE production (Fig. 6), although total CD90<sup>+</sup> T cells from the same splenocyte preparation strongly decreased IgE titres in the recipients. Likewise, the co-transfer of CD4<sup>+</sup> and CD8<sup>+</sup> T cells did not inhibit the development of the IgE response (Fig. 6). These findings prompted us to speculate that CD4<sup>+</sup>CD8<sup>+</sup> dnT cells might be involved in the regulation of IgE formation. We, therefore, purified CD4<sup>+</sup>CD8<sup>+</sup> dnT cells from K100 splenocytes, first by negatively selecting untouched T cells by the afore described method of indirect magnetic labelling, and then in a second step by depleting both

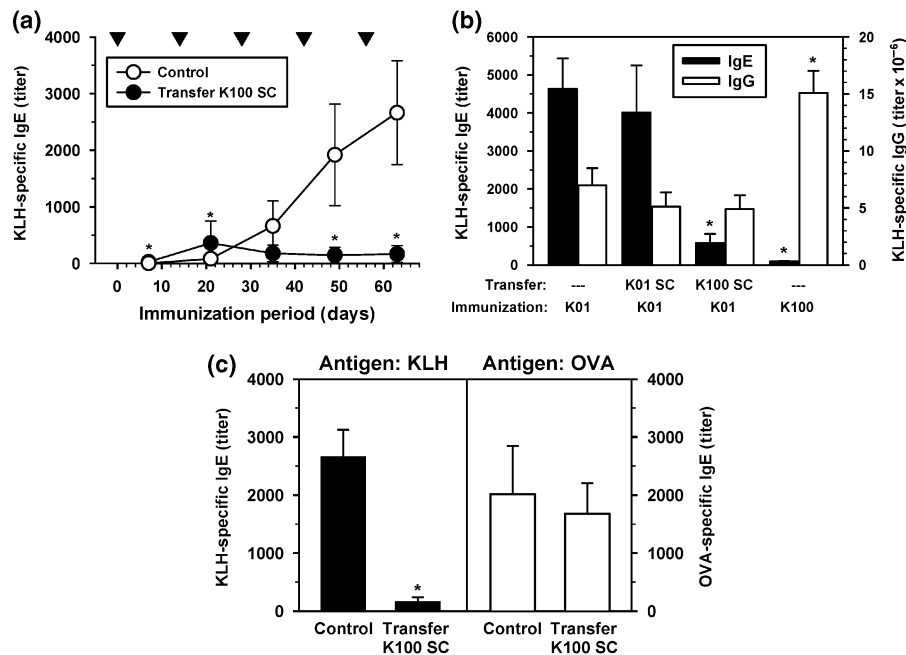


Fig. 5. Spleen cells from K100 mice suppress IgE production after adoptive transfer in an antigen- and isotype-specific manner. BALB/c mice were repeatedly immunized as described in the legend to Fig. 1. Seven to 14 days after the fifth immunization, K100 (a–c) or K01 (b) splenocytes (SC) were isolated and transferred into naïve BALB/c mice. Recipients and untreated control mice ( $n=4$ ) were immunized five times with  $0.1\text{ }\mu\text{g}$  keyhole limpet haemocyanin (KLH) (a–c) or  $10\text{ }\mu\text{g}$  ovalbumin (OVA) (c) adsorbed to Alum [indicated in (a) by black triangles]. Sera were taken as indicated (a) or 7–10 days after the fifth immunization (b, c) and levels of antigen-specific Ig were determined by ELISA. Data are presented as mean Ig titres  $\pm$  SEM of individual mice. \* $P<0.05$ , vs. control group. Graphs in (a) and (b) are representative of five and those in (c) of two independent experiments.

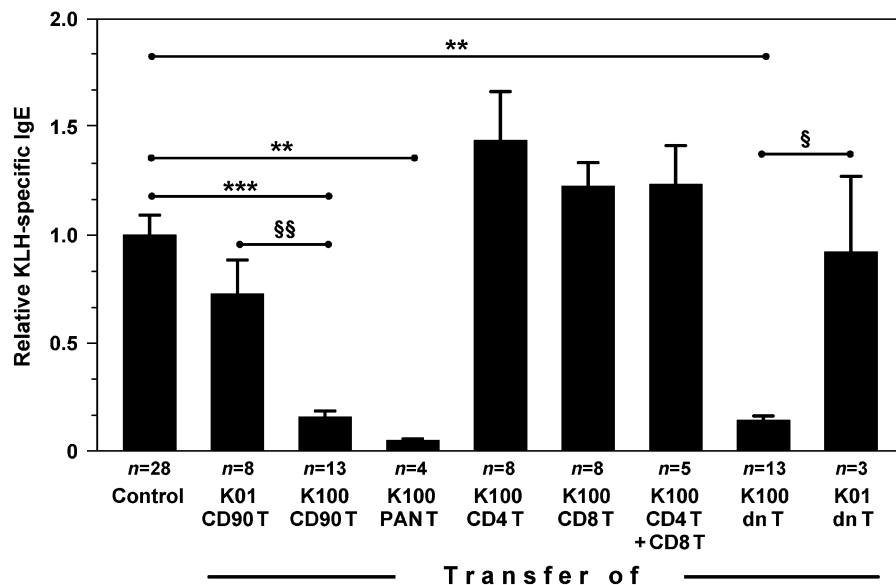


Fig. 6.  $\text{CD4}^-\text{CD8}^-$  double negative (dnT) cells are responsible for the suppression of IgE production after the adoptive transfer of K100 spleen cells. BALB/c mice were repeatedly immunized as described in the legend to Fig. 1. Seven to 14 days after the fifth immunization, positively (CD90 T) or negatively (PANT) selected total T cells, positively selected  $\text{CD4}^+$  (CD4 T) and/or  $\text{CD8}^+$  (CD8 T) T cells or  $\text{CD4}^-\text{CD8}^-$  dnT cells were transferred into naïve BALB/c mice. Recipients and untreated control mice were immunized five times with  $0.1\text{ }\mu\text{g}$  keyhole limpet haemocyanin (KLH) adsorbed to Alum. Sera were taken 7–10 days after the fifth immunization and KLH-specific IgE titres were determined by ELISA. Data were acquired in a total of seven independent experiments. In every experiment, IgE content in sera was normalized to the mean IgE content in the sera collected from mice of the respective control group without transfer. Data are presented as mean IgE content  $\pm$  SEM of individual mice (total numbers of control and recipient mice are indicated). \*\* $P<0.01$ , \*\*\* $P<0.001$ , vs. control group; § $P<0.05$ , §§ $P<0.01$ , transfer of K01 vs. K100 cells.

CD4<sup>+</sup> and CD8<sup>+</sup> T cells using direct magnetic labelling. In fact, mice that had received K100 CD4<sup>+</sup>CD8<sup>+</sup> dnT cells were significantly impaired in producing specific IgE after repeated immunization with low doses of KLH (Fig. 6), in contrast to control mice without cell transfer or mice that had received CD4<sup>+</sup>/CD8<sup>+</sup> T cells positively selected in the latter step of the isolation procedure (data not shown). The suppressive potential was attributable to CD4<sup>+</sup>CD8<sup>+</sup> dnT cells purified from K100 mice because the transfer of CD4<sup>+</sup>CD8<sup>+</sup> dnT cells from K01 mice resulted only in a marginal reduction of IgE titres (Fig. 6).

## Discussion

Production of IgE antibodies in humans and mice is highly dependent on the antigen dose the immune system has encountered. We and others have shown in mouse models of allergic hyperresponsiveness using clinically relevant allergens such as phospholipase A<sub>2</sub> [9, 31] and OVA [31, 32] or model protein antigens such as KLH [10] that repeated immunization of mice with low doses of antigen adsorbed to Alum as an adjuvant promoted the development of a strong antigen-specific IgE response. In contrast, antigen-specific IgE titres were marginal after consecutive immunization of mice with high antigen doses. So far, antigen dose-dependent differences in IgE production primarily were monitored by measuring the IgE content in sera of immunized mice using an ELISA-based detection system. However, this assay carries the inherent risk that an excess of IgG antibodies in the sera might interfere with the binding of the IgE antibodies to the antigen by competing for the same epitopes, thus merely masking the presence of IgE antibodies [33]. In this communication, we confirmed the antigen dose-dependent regulation of IgE formation also on a functional level by determining the anaphylactic activity of immune sera applying the RBL-2H3 mediator release assay. This assay indicates the FcεRI-mediated binding of IgE antibodies, subsequently triggering the antigen-dependent stimulation of RBL-2H3 cells. In line with our finding using the ELISA assay that IgE production after immunization with high antigen doses is decreased, the ability of K100 sera to provoke degranulation of RBL-2H3 basophils was significantly reduced as compared with K01 sera.

We have shown previously that B lymphocytes in mice immunized with low doses of KLH developed into IgE-secreting plasma cells, whereas in contrast B lymphocytes from animals primed with high doses of KLH were arrested on an IgG1-expressing differentiation stage *in vivo* [10, 30]. However, the regulatory mechanism controlling the antigen dose-dependent development of B cells into either IgG1- or IgE-producing plasma cells is yet unknown. It is widely accepted that the induction of IgE synthesis is facilitated by the polarization of T lymphocytes into Th2 cells. Accordingly, K01 splenocytes predominantly

released cytokines like IL-4, IL-5 and IL-13 after restimulation *in vitro*, but hardly any IFN-γ. In contrast, following immunization with high doses of KLH, the production of the Th2-associated cytokines was considerably impaired. However, the level of IFN-γ production by K100 splenocytes was not enhanced, as has also been reported before for CBA/J mice [34]. This observation is coherent with previous reports using low vs. high doses of OVA or PLA<sub>2</sub> for sensitization via the intraperitoneal route [31, 32]. In the present report, we provide further evidence that antigen dose-dependent differences in IgE antibody production are not due to differential polarization towards Th1 and Th2 cell subsets. We demonstrate that K100 IFN-γ<sup>-/-</sup> and K100 IL-12p40<sup>-/-</sup> mice, which due to a genetic defect are not capable of synthesizing bioactive IFN-γ or IL-12 and thus do not mount Th1 immune responses [17, 18], did not exhibit substantial IgE titres in their sera whereas their K01 counterparts did produce reliable quantities of IgE as efficiently as C57BL/6 wild-type mice did. Altogether, these data argue against the assumption that immunization with high doses of antigen generates Th1 cells, which interfere with the induction of IgE responses.

In this report, we verified the observation that splenocytes of mice repeatedly immunized with high antigen doses are hyporeactive after antigenic restimulation *in vitro* as compared with splenocytes from mice primed with low antigen doses [31, 32]. Suppression of antigen-driven proliferative and cytokine responses, as observed in K100 splenocyte cultures, are characteristics of peripheral T cell tolerance, which for example has also been shown to be established in the course of allergen-specific SIT. In SIT, the induction of a tolerant state has been described to be predominantly attributed to the activation of allergen-specific Tregs [4]. This led us to speculate that distinct Treg subpopulations might be more frequent among splenocytes from K100 mice. Therefore, we determined the frequency of thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> nTregs, which have been associated with the spontaneous remission of cow's milk allergy in tolerant children [35]. These children exhibited higher frequencies of circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells but lower allergen-specific proliferative responses of peripheral blood mononuclear cells *in vitro* than children who developed clinically active allergy. CD4<sup>+</sup>CD25<sup>+</sup> nTregs have also been shown to inhibit allergic responses to inhaled antigens [36]. *In vitro* CD4<sup>+</sup>CD25<sup>+</sup> nTregs suppress the activation and proliferation of effector T cells in a cell-cell contact-dependent manner [37]. However, our analysis revealed that the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells coexpressing CTLA-4 or GITR, two molecules constitutively present on the surface of CD4<sup>+</sup>CD25<sup>+</sup> nTregs [21, 22], or Foxp3, which is the key transcription factor in the development of CD4<sup>+</sup>CD25<sup>+</sup> nTregs [23], was not significantly different among K01 and K100 splenocytes.

An alternative explanation for tolerance induction in K100 mice would be the activation of adaptive Tregs,

which mainly operate via the secretion of soluble mediators with suppressive capacity such as the immunomodulatory cytokines IL-10 and TGF- $\beta$ , respectively [38]. In healthy non-allergics as well as in allergen-sensitized but tolerant individuals, IL-10- and/or TGF- $\beta$ -producing T regulatory type 1 (Tr1) cells counterbalance the activity of allergen-specific Th2 cells and thus prevent the initiation of clinical symptoms [6, 7, 39]. In addition, the skewing of allergen-specific T cells towards a Tr1 cell phenotype appears to be a pivotal event in successful SIT [39, 40]. However, we did not observe an enhanced production of TGF- $\beta$ 1 or IL-10 by K100 splenocytes, suggesting that Tr1 cells do not play a major role in tolerance induction and inhibition of IgE responses after immunization with high antigen doses. Moreover, other T cell populations such as  $\gamma\delta$  T cells or CD8<sup>+</sup> T cells, whose suppressor cell function for IgE production and allergic sensitization has been controversially discussed [41, 42], were not found to be more frequent among K100 splenocytes.

The notion that neither CD4<sup>+</sup> T cells nor CD8<sup>+</sup> T cells included the putative suppressor cell population is supported by the results of the transfer experiments, which revealed an inhibitory potential of splenocytes and whole T cells from K100 mice on IgE production after injection into naïve recipients, but not of isolated CD4<sup>+</sup> or CD8<sup>+</sup> T cells. Actually, we defined CD4<sup>+</sup>CD8<sup>+</sup> dnT cells as a T cell subpopulation, which considerably suppressed the development of an IgE response. Until now, CD4<sup>+</sup>CD8<sup>+</sup> dnT cells have been described not only as suppressor T cells mainly in animal models of tolerance induction after allogeneic or xenogeneic transplantation, but also in experimental murine studies to prevent graft-vs.-host disease or to control autoimmune T cell responses in mice [43–46]. Recently, a CD4<sup>+</sup>CD8<sup>+</sup> dnT cell population with similar functional properties has also been described in humans [47]. However, a regulatory role for CD4<sup>+</sup>CD8<sup>+</sup> dnT cells in IgE immune responses or type I hypersensitivity has not been documented so far.

It has to be further elucidated whether the CD4<sup>+</sup>CD8<sup>+</sup> dnT cells identified are related to one of the aforescribed suppressor T cells and whether they follow similar differentiation pathways. Because of the different capacities of CD4<sup>+</sup>CD8<sup>+</sup> dnT cells purified from KO1 and K100 mice to control IgE formation after transfer, a comparative molecular analysis of these cells might provide an insight into the effector mechanisms exerted by CD4<sup>+</sup>CD8<sup>+</sup> dnT cells, induced by immunization with high antigen doses, on the humoral immune response.

In summary, we have shown for the first time that immunization with high antigen doses selectively activated CD4<sup>+</sup>CD8<sup>+</sup> dnT cells, which acted as antigen-specific suppressor cells for IgE production. On the basis of these data, it is tempting to speculate that in humans CD4<sup>+</sup>CD8<sup>+</sup> dnT cells might also play a role in the induction and maintenance of high-dose tolerance against

allergens and that these cells, in addition to conventional IL-10- or TGF- $\beta$ -producing regulatory T cells, are generated/stimulated in the course of causative therapeutic treatments of type I hypersensitivities such as SIT.

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