European Journal of Immunology

SHORT COMMUNICATION

Preexisting antigen-specific immune responses are modulated by oral KLH feeding in humans

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Oral tolerance is the antigen-specific inhibition of a systemic immune response after oral antigen uptake and well established in animal models. We recently showed that keyhole limpet hemocyanin (KLH) feeding modulates subsequently induced systemic immune responses in humans as well. In the present study, we investigated whether oral KLH can also modulate preexisting antigen-specific systemic B- and T-cell responses. We induced delayed-type hypersensitivity (DTH) reactions as well as systemic KLH-specific B- and T-cell responses by subcutaneous KLH injections. Subsequent oral KLH administration decreased the small proportion of antigen-specific $CD4^+$ T cells positive for the cytokine IL-17 at the end of the feeding regimen even further. After reimmunization, there was no difference in DTH reactions and the KLH-specific B-cell responses, but KLH-fed volunteers had an increased proportion of antigen-specific $CD4^+$ T cells positive for IL-10 and a reduced proportion of antigen-specific $CD4^+$ T cells positive for the skin-homing receptor cutaneous lymphocyte antigen and IL-2 and IFN-γ. Taken together, oral KLH can modulate a preexisting systemic KLH-specific immune response. These results suggest that feeding antigen may offer therapeutic strategies for the suppression of unwanted immune reactions in humans.

Keywords: B- and T-cell response · Cytokines · Human · KLH feeding · Tolerance/immune modulation

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Introduction

Systemic cellular and/or humoral immune responses can be downregulated in an antigen-specific manner by feeding with protein antigen, a phenomenon known as oral tolerance [1]. Obviously oral tolerance offers a therapeutical approach for the inhibition of autoimmune-inflammatory diseases, and has been investigated in experimental autoimmune diseases in mice [2]. However, responses to oral antigen differ significantly between species and clinical studies are rare [3, 4]. Human studies for the treatment of autoimmune diseases exploiting oral tolerance (e.g. diabetes, multiple sclerosis, or rheumatoid arthritis) have been initiated but shown limited efficacy so far [2, 5]. Successful induction of tolerance in humans against the neoantigen keyhole limpet hemocyanin (KLH) has been described in healthy volunteers [6]. However, previous findings suggested that oral KLH feeding in

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humans may be unable to suppress a preexisting immune response to KLH [7] and little is known about modulatory effects of oral antigen administration apart from priming and tolerance.

We recently demonstrated a novel effect of oral immune modulation in humans by showing that oral KLH on its own can induce a systemic antigen-specific response with a bias toward a Th type 2 cytokine pattern [8]. Further, oral KLH primed a subsequent systemic immune response after parenteral immunization and shifted the cytokine pattern of KLH-specific T cells toward a Th type 2 phenotype and amplified KLH-specific B-cell responses [8].

In the present study, we sought to determine whether oral administration of KLH can also modulate preexisting antigen-specific systemic immune responses to KLH. We addressed this issue by analysis of the effects of oral KLH application to volunteers who already had established a systemic immune response after prior parenteral KLH immunization. Immune reactions were analyzed after the primary immunization, after oral antigen application and after a subsequent reimmunization. In comparison of the oral group with the control group, which did not receive oral KLH, the delayed-type hypersensitivity (DTH) reactions after KLH priming and booster appeared unaffected and proliferation of $CD4^+$ T cells remained unchanged. Nevertheless oral KLH increased the proportion of KLH-specific $CD4^+$ T cells producing IL-10 and reduced the proportion of KLH-specific $CD4^+$ T cells producing IL-2, IL-17, and IFN-γ compared to controls. KLH-specific B-cell responses remained unaltered.

Results and discussion

Oral KLH modulated preexisting systemic KLH-specific $CD4^+$ T-cell responses

According to the protocol, subjects of the oral and the control group were immunized with 1 mg KLH (subcutaneously and intradermally) to induce a systemic immune response. KLH-specific $CD4^+$ T cells were quantified as proportion of $CD154^+$ $CD4^+$ T cells after stimulation with an agonistic anti-CD28 mAb and KLH corrected for the proportion of $CD154^+$ $CD4^+$ T cells after stimulation with anti-CD28 alone (Fig. 1).

As expected the DTH reactions were not different (data not shown) and the frequencies as well as the phenotype in terms of cytokine production, homing marker expression, and proliferation of KLH-specific $CD4^+$ T cells were similar in both groups (Fig. 2).

One week after second priming, 5 mg KLH was fed in the oral group for 10 days. On day 24 (i.e. the last day of feeding regimen) in the oral group compared to the controls, the proportion of KLH-specific $CD4^+$ T cells producing IL-17 was decreased indicating a lowered proinflammatory immune response (Fig. 2). Because the proportion of $CD4^+$ T cells expressing IL-17 was very low, we used PBMC from day 24 stored in liquid nitrogen that were available from two volunteers each of the control and the oral group to measure IL-17 concentration in culture supernatants after stimulation with KLH and anti-CD28. In accordance with the cytofluorometric data KLH-induced IL-17 secretion was much lower in PBMC from volunteers who had received oral KLH (Fig. 2, inset).

At day 35 of the experimental regimen, that is 11 days after the end of KLH feeding and 28 days after the second priming with KLH, all participants were boosted with 0.1 mg KLH (subcutaneously and intradermally). Oral KLH did not suppress DTH reactions that were similar in both groups, nor reduce the KLH-specific proliferation of $CD4^+$ T cells or the frequencies of KLH-specific $CD154^+$ $CD4^+$ T cells. However, KLH feeding had a profound impact on the phenotype of KLH-specific T cells. In the oral group compared to controls we found a higher proportion of antigen-specific $CD4^+$ T-cells positive for IL-10 and lower proportions of KLH-specific $CD4^+$ T cells positive for the skin-homing receptor cutaneous lymphocyte antigen (CLA), for IL-2 and for IFN-γ at day 35 of the experimental regimen (Fig. 2). These differences were only transient, however, and only the proportion of antigen-specific $CD4^+$ T cells positive for IL-10 remained increased at day 42, that is 7 days after reimmunization and 18 days after the end of oral KLH (Fig. 2).

In summary, our findings provide evidence that repeated oral low-dose KLH modulates a preexisting immune response by skewing the proportion of cytokine producers within the KLH-specific $CD4^+$ T cells toward more IL-10- and less IFN-γ-, IL-2-, IL-17-, and CLA-positive cells (Fig. 2). Thus, oral KLH leads to a less inflammatory phenotype in KLH-specific $CD4^+$ T cells.

Preexisting systemic KLH-specific B-cell responses are not affected by oral KLH

To investigate effects of oral KLH on preexisting KLH-specific B-cell responses, we compared KLH-specific serum antibodies in both groups. We found no significant effect of our feeding regimen on the amounts of KLH-specific IgA, IgG, IgM, IgG1, IgG2, IgG3, and IgG4 levels after priming, feeding, and boost with KLH, respectively (Fig. 2). This is in accordance with previous findings in human oral tolerance utilizing KLH [7] and indicates that oral immunization by KLH prior to systemic immunization [8] is more effective for the modulation of B-cell responses. This could be due to an early effect on Th cells that play a pivotal role in the development of antigen-specific B-cell responses against protein antigens and are probably required in the selection, expansion, and differentiation of KLH-specific B cell [9].

Effects of oral antigen administration in humans

Oral tolerance is the antigen-specific inhibition of a systemic immune response and therefore offers a very attractive approach to treat autoimmune diseases by oral application of the causative auto-antigen. The mechanism of oral tolerance is well established in rodents [2]. In humans it has been reported that oral application of KLH in healthy humans reduces the DTH reaction and lowers KLH-specific T-cell proliferation in the peripheral blood after subsequent systemic immunization, which are two key parameters

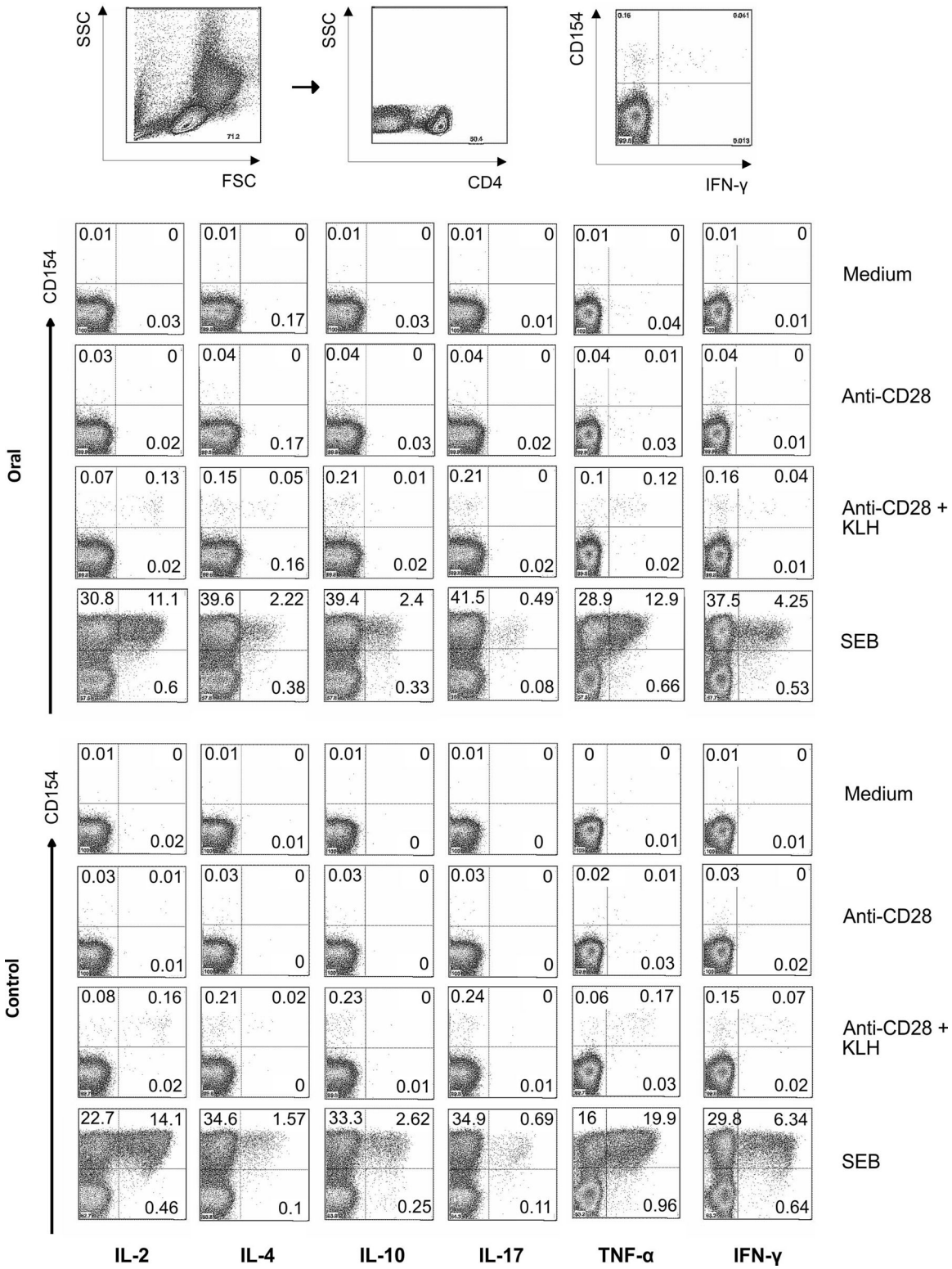

Figure 1. KLH-specific cytokine expression of CD4$^+$ T cells. KLH-specific CD4$^+$ T cells were identified by the expression of CD154 after in vitro restimulation with KLH and anti-CD28 using the gating strategy shown in the upper panel. Expression of cytokines by KLH-reactive cells in the oral versus the control group was quantified as percentage of cytokine-producing CD154$^+$ CD4$^+$ T cells after combined KLH and anti-CD28 stimulation minus percentage of cytokine-producing CD154$^+$ CD4$^+$ T cells after anti-CD28 stimulation. Representative dot plots for IL-2, IL-4, IL-10, IL-17, TNF-α, and IFN-γ without and after stimulation with anti-CD28, KLH combined with anti-CD28 and SEB at day 35 are shown. Data are representative of eight independent experiments.

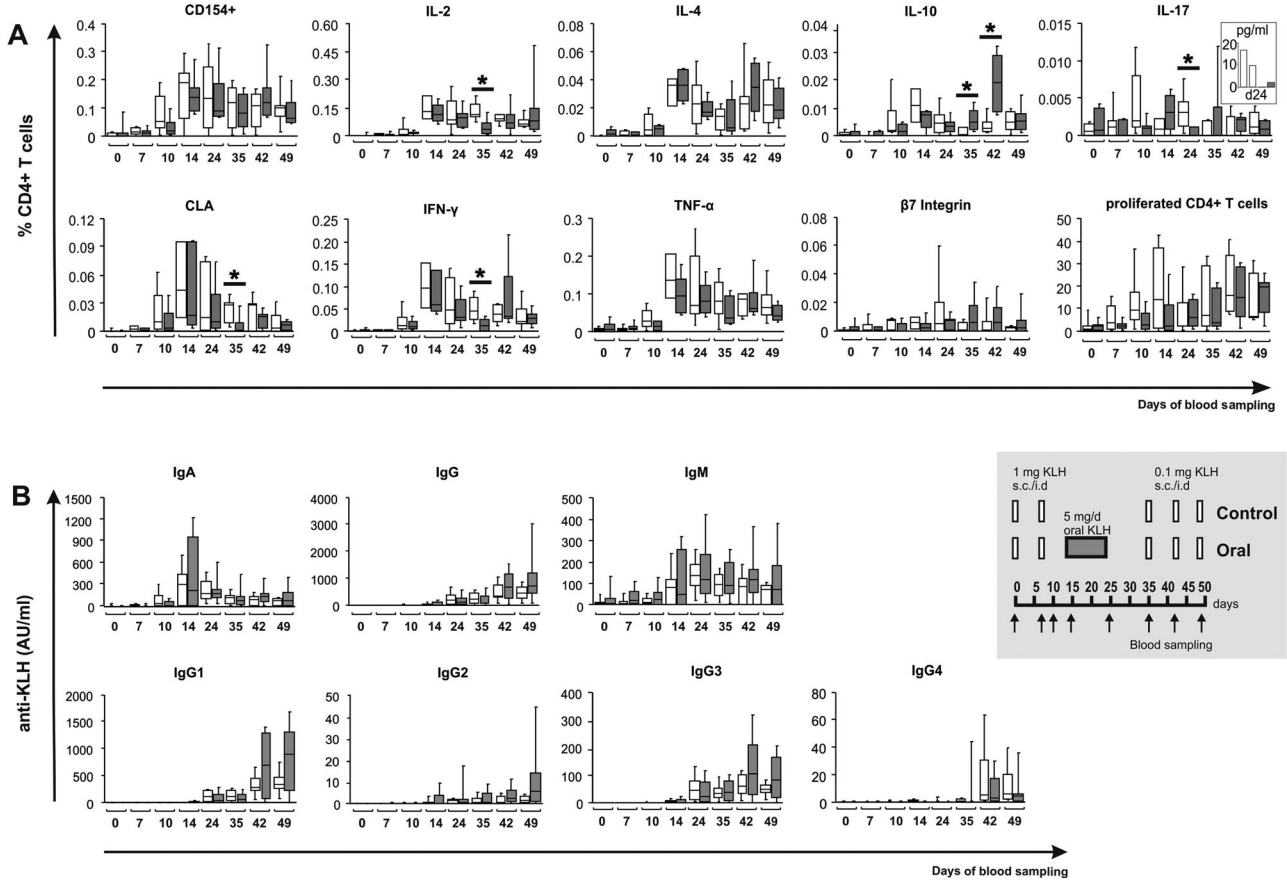

Figure 2. Effects of oral KLH on KLH-specific $CD4^+$ T-cell and B-cell responses induced by previous parenteral immunization. (A and B) Blood and sera from subjects of the oral ($n = 8$, gray) and the control group ($n = 8$, white) were collected at days 0, 7, 10, 14, 24, 35, 42, 49. (A) KLH-specific $CD4^+$ T cells were analyzed by flow cytometry. Shown are the percentages of KLH-specific $CD154^+$ cells, the percentages of cells expressing IL-2, IL-4, IL-10, IL-17, IFN-γ, TNF-α, CLA, and β7 integrin together with CD154 as well as the percentage of proliferated cells within the $CD4^+$ T-cell population. Inset: At day 24 PBMCs of two participants from the oral and the control group were cultured for 3 days with KLH and anti-CD28 mAb or with anti-CD28 mAb alone and the IL-17 concentration in the culture supernatants was measured by cytometric bead array. (B) Sera were analyzed for KLH-specific antibodies as described in the Materials and methods. Kinetics of KLH-specific antibody responses are shown. The amounts of KLH-specific antibodies are denoted as arbitrary units, because no specific anti-KLH control antibodies are available. (A and B) Data are shown as the median, 25th and the 75th percentile (boxes) and minimum and maximum values (whiskers) of eight donors per group. $^*p < 0.05$; Mann–Whitney test.

of oral tolerance. The KLH-specific humoral immune response is unaltered or even amplified by KLH feeding [6, 10]. Similar findings have been reported from various experimental studies of oral tolerance in animal models [2].

However, treatment of autoimmune diseases by auto-antigen feeding would probably take place in most cases after the onset of the autoimmune reaction. In the current study, we therefore analyzed the effects of oral KLH on an existing systemic immune response. Furthermore, the outcomes of oral antigen administration appear to be dose-dependent at least in mice: a single high dose of oral antigen induces antigen-specific T-cell inactivation or deletion, whereas repeated low dose feeding of oral antigen favor the generation of active suppression by regulatory T cells secreting anti-inflammatory cytokines like IL-10 [11, 12]. In our study, we applied comparatively small amounts of oral KLH. The proportions of cytokine producing cells were often very low, and differences should therefore be interpreted with caution. However, we could confirm the difference in IL-17 producing cells by measurements in culture supernatants of the PBMC available from day 24. Thus, the reduced proportions of antigen-specific cells producing IL-2, IL-17, and IFN-γ found in our study suggest a downregulated proinflammatory immune response by oral KLH toward a rather anti-inflammatory immune response as indicated by the increased proportion of antigen-specific IL-10-producers. Whether this is caused either by preexisting or induced regulatory T cells and if it is accompanied by TGF-β production must be clarified in further studies.

Activation of T cells by DC imprints their tissue-specific homing potential. Skin homing T cells are induced by DC from peripheral lymph nodes [13, 14] whereas gut-homing T cells are induced by DC from Peyer's patches or MLN [15, 16]. The selective capacity of murine intestinal DC to generate integrin $\alpha4\beta7^+$ $CCR9^+$ gut-homing T cells involves retinoic acid and seems to be restricted to the specialized subset of $CD103^+$ MLN DC originating from the

small intestinal lamina propria [17]. A similar mechanism could explain the reduced expression of the skin-homing receptor CLA on KLH-specific T cells after oral KLH feeding (i.e. at day 35).

Some data from human and animal studies provided strong evidence for suppression of preexisting immune responses by subsequent mucosal antigen administration [18–20]. However, antigen feeding did not necessarily result in the improvement of the disease outcome [5, 21]. Recently, oral KLH administration to healthy volunteers failed to suppress a preexisting systemic B and T-cell immune response to KLH [7]. Thus, effects of oral administration of antigen and the efficiency in inducing oral tolerance appear to depend on application regimen. Timing and doses of oral antigen also seem to have a profound impact. In this regard, we recently demonstrated that low-dose oral KLH in humans led to amplified B-cell responses and shifted the cytokine pattern of KLH-specific $CD4^+$ T cells from a Th type 1 toward a Th type 2, thereby suggesting a novel effect of oral immune modulation [8].

Our findings presented in the current study revealed an anti-inflammatory, immune modulating effect of low-dose oral KLH on an existing systemic immune response. Thus, extended protocols to prolong or amplify this effect can clarify the underlying cellular and/or molecular mechanisms and its therapeutical potential.

Concluding remarks

Our data demonstrate that low dose oral antigen can modulate a preexisting systemic immune response in humans toward a rather anti-inflammatory immune response. Although we did not observe classical oral tolerance, the observed shift supports the possibility of treatment of autoimmune and inflammatory diseases utilizing amended protocols.

Materials and methods

Keyhole limpet hemocyanin

Keyhole limpet hemocyanin (KLH, Immucothel®) from Biosyn (Fellbach, Germany) was fed incorporated in acid-resistant capsules, which were produced under GMP conditions by Rentschler Pharma (Laupheim, Germany).

Subjects and study design

The study was approved by the local ethics committee and informed written consent was obtained from all participants. The oral group (oral, four males, four females; mean age 33 years, range 21–53) and the control group (controls, three males, five females; mean age 36 years, range 24–55) were parenterally immunized with 0.5 mg KLH intradermally and 0.5 mg KLH subcutaneously on days 0, 7, 35 as shown in Fig. 2. Members of the oral group ingested 5 mg KLH in acid-resistant capsules from day 14 to day 24 (Fig. 2). At the indicated timepoints 50 mL of heparinized blood samples were obtained.

Cell isolation and stimulation

PBMC were purified by density gradient centrifugation and KLH-specific $CD4^+$ T cells were identified through short-term stimulation of 1×10^6/mL PBMC with 1 μg/mL anti-CD28 (L293, BD Biosciences, Heidelberg, Germany) with or without 200 μg/mL KLH, medium, and *Staphylococcus* enterotoxin B (7 μg/mL, Sigma-Aldrich, Taufkirchen, Germany) as described by Kapp et al. [8]. Supernatants were collected from PBMC thawed from liquid nitrogen storage and cultured at a concentration of 1×10^6 PBMC/mL for 3 days with KLH and anti-CD28 mAb or with anti-CD28 mAb alone.

Flow cytometry

Flow cytometry and data analysis were performed as described by Kapp et al. [8] using anti-CD4 (SK3), anti-CD25 (2A3), anti-CD154 (TRAP1), anti-IFN-γ (B27), anti-IL-4 (MP4-25D2), anti-TNF-α (MAb11), anti-IL-10 (JES3-19F1), anti-IL-2 (MQ1-17H12), anti-CLA (HECA-452), anti-integrin β7 (FIB504), all from BD Biosciences; anti-CD19 (HIB19), anti-CD14 (61D3), anti-IL-17A (eBio64DEC17), and anti-CD62P (AK-4), all from eBioscience (San Diego, CA, USA). A total of 1×10^5 $CD4^+$ T cells were acquired with a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). For the quantification of KLH-specific $CD4^+$ T cells the proportion of $CD154^+$ $CD4^+$ cells after stimulation with anti-CD28 and KLH was corrected for the proportion of $CD154^+$ $CD4^+$ cells after stimulation with anti-CD28 alone. $CD154^+$ $CD4^+$ cells showed no relevant background in control fluorescence-minus-one staining (Supporting Information).

KLH-specific $CD4^+$ T-cell proliferation was measured as described earlier [8] after 7 days of in vitro stimulation with the CellTrace™ CFSE Cell Proliferation kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

IL-17 concentration in cell culture supernatants was analyzed with IL-17 enhanced cytometric bead array (BD Biosciences) according to the manufacturer's protocol.

Quantification of KLH-specific serum antibodies

ELISA of KLH-specific serum antibody were performed as described before [8] using biotinylated anti-IgA (G20-359), anti-IgM (G20-127), anti-IgG1 (G17-1), anti-IgG2 (G18-21), anti-IgG4 (JDC-14), anti-IgE (G7-26), all from BD Biosciences; anti-IgG (HP6017), anti-IgG3 (HP6050), both from Sigma-Aldrich. Absorbance was measured at 450 nm on a microplate reader (Tecan, Crailsheim, Germany). A reference serum containing pooled high-titer sera from immunized subjects were used as

reference serum that was defined to contain 1000 arbitrary units of KLH-specific IgA, IgG, and IgM. IgG subclasses within the reference serum were determined using the total IgG standard curve.

Statistical analysis

The Mann–Whitney test was used to analyze differences between unpaired data and p values < 0.05 were considered significant. Selected data are presented as box plots that show medians, 25th and 75th percentiles, and minimum and maximum values.

Acknowledgements: We thank Ulrike Dethlefs, Sylvia Münchow, Margarete Grabowski, and Yvonne Dell for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft grant SFB 633. This work is dedicated to Martin Zeitz (1950–2013).

Conflict of Interest: The authors declare no financial or commercial conflict of interests.

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Abbreviations: **CLA**: cutaneous lymphocyte antigen · **DTH**: delayed-type hypersensitivity · **KLH**: keyhole limpet hemocyanin

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Received: 16/7/2014
Revised: 12/3/2015
Accepted: 30/3/2015
Accepted article online: 1/4/2015

