

Modulation of systemic antigen-specific immune responses by oral antigen in humans

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Oral antigen uptake can induce systemic immune responses ranging from tolerance to immunity. However, the underlying mechanisms are poorly understood, especially in humans. Here, keyhole limpet hemocyanin (KLH), a neoantigen which has been used in earlier studies of oral tolerance, was fed in a repeated low-dose and a single high-dose protocol to healthy volunteers. KLH-specific CD4⁺ T-cell proliferation and cytokine production, as well as KLH-specific serum Ab and the effects of oral KLH on a subsequent parenterally induced systemic immune response, were analyzed. Repeated low-dose oral KLH alone induced antigen-specific CD4⁺ T cells positive predominantly for the gut-homing receptor integrin $\beta 7$ and the cytokines IL-2 and TNF- α ; some CD4⁺ T cells also produced IL-4. Oral feeding of KLH accelerated a subsequent parenterally induced systemic CD4⁺ T-cell response. The cytokine pattern of KLH-specific CD4⁺ T cells shifted toward more IL-4- and IL-10- and less IFN- γ , IL-2- and TNF- α -producing cells. The parenterally induced systemic KLH-specific B-cell response was accelerated and amplified by oral KLH. The impact of single high-dose oral KLH on antigen-specific immune responses was less pronounced compared with repeated low-dose oral KLH. These findings suggest that oral antigen can effectively modulate subsequently induced systemic antigen-specific immune responses. Immunomodulation by oral antigen may offer new therapeutic strategies for Th type1-mediated inflammatory diseases and for the development of vaccination strategies.

Key words: Cytokines · Human · Th1/Th2 cells · Tolerance/Suppression/Anergy · Vaccination



Supporting Information available online

Introduction

The immunological consequences of oral antigen uptake are highly variable and include the induction of secretory IgA

responses, systemic immunity and even systemic tolerance [1, 2]. Pathogens recognized by mucosal antigen presenting cells as “danger signals” (e.g. by Toll-like receptors) lead to inflammatory conditions and favor the development of cellular and humoral immune responses [3]. On the contrary, soluble, nonadherent antigens fed to mice usually suppress systemic

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cellular and/or humoral immune responses, a phenomenon known as oral tolerance [1, 4–7]. Systemic tolerance in these models may result from a variety of effects including clonal anergy, clonal deletion and active regulation [2]. Understanding the mechanisms leading to such diverse outcomes after mucosal antigen uptake is not only important for mucosal vaccination [3, 7], but may also offer new options for immunomodulation or the treatment of autoimmune diseases [8–10]. Unfortunately, responses to oral antigen differ significantly between species [8, 11], and human studies are rare.

Similar to mouse models, systemic immune responses can be induced in humans by oral uptake of pathogens which invade the mucosa, e.g. *Salmonella typhi* Ty21a [12, 13], or of toxins which can bind to mucosal epithelial cells and compromise the mucosal barrier, e.g. cholera toxin [14, 15].

The induction of human oral tolerance has been described [16–18] after feeding keyhole limpet hemocyanin (KLH), a neoantigen which is safe to use in humans. Compared with rodent studies, the antigen doses used were much lower and effects observed were not as uniform: reduced delayed-type hypersensitivity (DTH) [17, 18] and antigen-specific proliferation of PBMC [16–18] have been reported after feeding KLH, although KLH-specific B-cell responses were unaffected [16] or even amplified [17, 18].

Functional Th-cell subsets are believed to determine the type of immune responses, but have not been examined after oral antigen challenge in humans. The frequencies of human antigen-specific Th cells or even T-regulatory cells responding to a specific antigen are usually low, ranging from 1:1000 to 1:1 000 000 [19]. We used antigen-reactive CD154 expression which allows the detection of antigen-specific CD4⁺ T-effector cells after short time *in vitro* stimulation [20] to analyze antigen-specific effector Th cells after oral feeding of KLH. Markers for antigen-specific tolerized or regulatory T cells are not available, but oral tolerance induction should be evident by comparison of antigen-specific Th-cell responses after parenteral immunization in volunteers with and without prior oral KLH.

In this report, we demonstrate a novel effect of oral immunomodulation in humans: we show that oral KLH can induce a systemic antigen-specific Th-cell response with a bias toward a Th type 2 cytokine pattern. Furthermore, oral KLH primes a subsequent systemic immune response induced by parenteral immunization, shifts the cytokine pattern of KLH-specific cells toward a Th type 2 and amplifies KLH-specific B-cell responses.

Results

Oral KLH induces systemic KLH-specific CD4⁺ T-cell responses

Healthy volunteers ingested 5 mg KLH in acid-resistant capsules from day 1 to day 10 (repeated low-dose oral group, oral_{RDL}) or a single dose of 50 mg KLH at day 1 (single high-dose oral group, oral_{SHD}, Fig. 1A). KLH-specific CD4⁺ T cells were identified by

the expression of CD154 after short-term *in vitro* stimulation with KLH in combination with the costimulatory Ab anti-CD28 [20].

Prior to oral antigen administration, the frequencies of CD154⁺CD4⁺ T cells did not differ after stimulation with KLH and anti-CD28 or stimulation with anti-CD28 alone; at day 21, i.e. 11 days after the last oral antigen dose, the frequencies of CD154⁺CD4⁺ T cells were higher after stimulation with KLH and anti-CD28 than after stimulation with anti-CD28 alone suggesting induction of KLH-specific CD4⁺ T cells by oral KLH (Fig. 1B). Compared with day 0, the frequencies of CD154⁺CD4⁺ T cells were increased at day 21 in the oral_{RDL} group (Fig. 1A). In fact, six of the eight subjects in the oral_{RDL} group, but only one of the seven subjects in the oral_{SHD} group, showed an at least threefold increase of CD154⁺CD4⁺ T cells at day 21 compared with day 0 and were therefore considered as responders (Fig. 1A).

Next, we analyzed the expression of cytokines and homing receptors within the KLH-specific CD4⁺ T-cell population at day 21, e.g. prior to the first parenteral immunization (Fig. 1C). In the oral_{RDL} group, antigen-specific CD4⁺ T cells produced predominantly IL-2 and TNF- α . Interestingly, the proportion of IL-4-producing cells was significantly higher compared with IFN- γ -producing cells. IL-10⁺ cells were rare. Antigen-specific CD154⁺CD4⁺ T cells mainly expressed the gut-homing receptor integrin β 7 and not the skin-homing receptor cutaneous lymphocyte antigen (CLA). Compared with the oral_{RDL} group, KLH-specific CD4⁺ T cells in the one responder of the oral_{SHD} group also rarely expressed IL-10 or CLA, but showed lower frequencies of IL-4, IL-2, TNF- α and integrin β 7 expression, although IFN- γ -producing cells were common (Fig. 1C).

It has been shown that the frequency of antigen-specific Th cells in peripheral blood is low, even after immunization [19–21]. We therefore confirmed a KLH-specific increase in CD154⁺CD4⁺ T cells expressing the cytokines investigated or integrin β 7 at day 21 in the oral_{RDL} group by comparison with control cultures restimulated with anti-CD28 alone (Supporting Information Fig. 1B).

Oral low-dose KLH accelerates the specific CD4⁺ T-cell response to subsequent parenteral immunization

Following oral KLH administration, the subjects of the oral_{RDL} and oral_{SHD} groups were immunized parenterally (subcutaneously and intradermally) with KLH to induce a systemic immune response. The control subjects were only parenterally immunized. A similar DTH reaction was observed in both the oral groups and the control group after parenteral immunization. Compared with controls, CD4⁺ T-cell proliferation in the oral_{RDL} and oral_{SHD} group was significantly increased following parenteral immunizations at days 21 and 28 (Fig. 2A and B).

In the control group, KLH-specific CD4⁺ T cells appeared at day 32, i.e. 4 days after the second immunization and reached a maximal frequency at day 35. Similar kinetics were seen for all subpopulations of KLH-specific CD4⁺ T cells, except

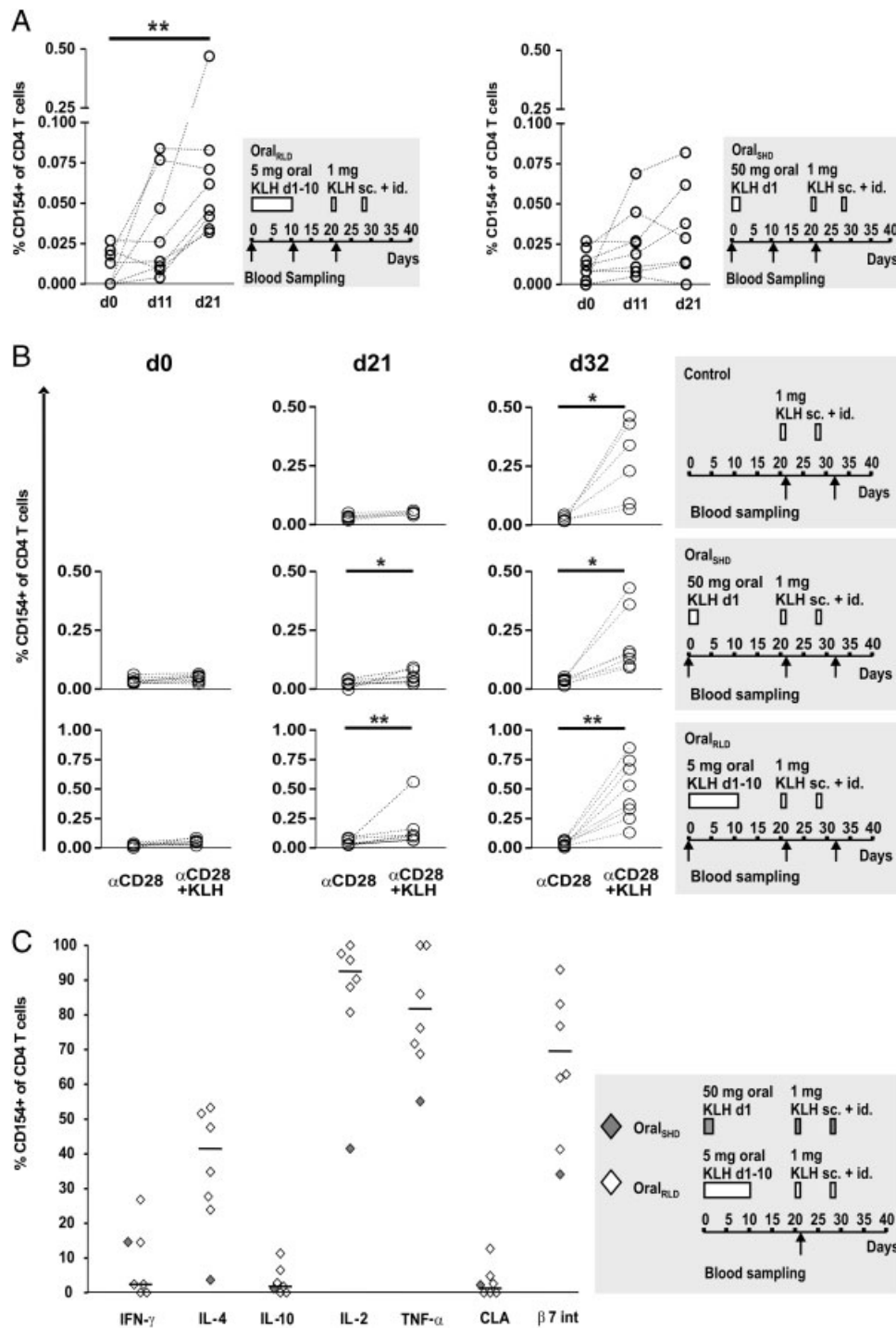


Figure 1. Oral KLH induces KLH-specific systemic CD4⁺ T-cell responses. PBMC were isolated before and after repeated low-dose (oral_{RLD}) or single high-dose (oral_{SHD}) oral KLH, stimulated *in vitro* with KLH and anti-CD28 or anti-CD28 alone, and analyzed by flow cytometry. CD154⁺CD4⁺ cells were considered KLH specific. Parenteral immunization was done in the upper arm with 0.5 mg KLH intradermally and 0.5 mg KLH s.c. (A) Percentages of KLH-specific CD4⁺ T cells at days 0, 11 and 21 after repeated oral KLH low dose (oral_{RLD}, *n* = 8, left) and single oral KLH high dose (oral_{SHD}, *n* = 7, right). (***p* < 0.01, Wilcoxon matched pairs test). (B) Comparison of the frequencies of CD154⁺ CD4⁺ T cells after stimulation with KLH and anti-CD28 or anti-CD28 alone at days 0, 21 and 32 in the oral_{RLD}, oral_{SHD} and control group (***p* < 0.01, **p* < 0.05, Wilcoxon matched pairs test). (C) Expression of cytokines and homing receptors within the KLH-specific CD4⁺ T-cell population at day 21 for the responding individuals (six oral_{RLD} and one oral_{SHD}) with an at least threefold increase of KLH-specific CD4⁺ T cells compared with day 0. Individual data and medians are indicated.

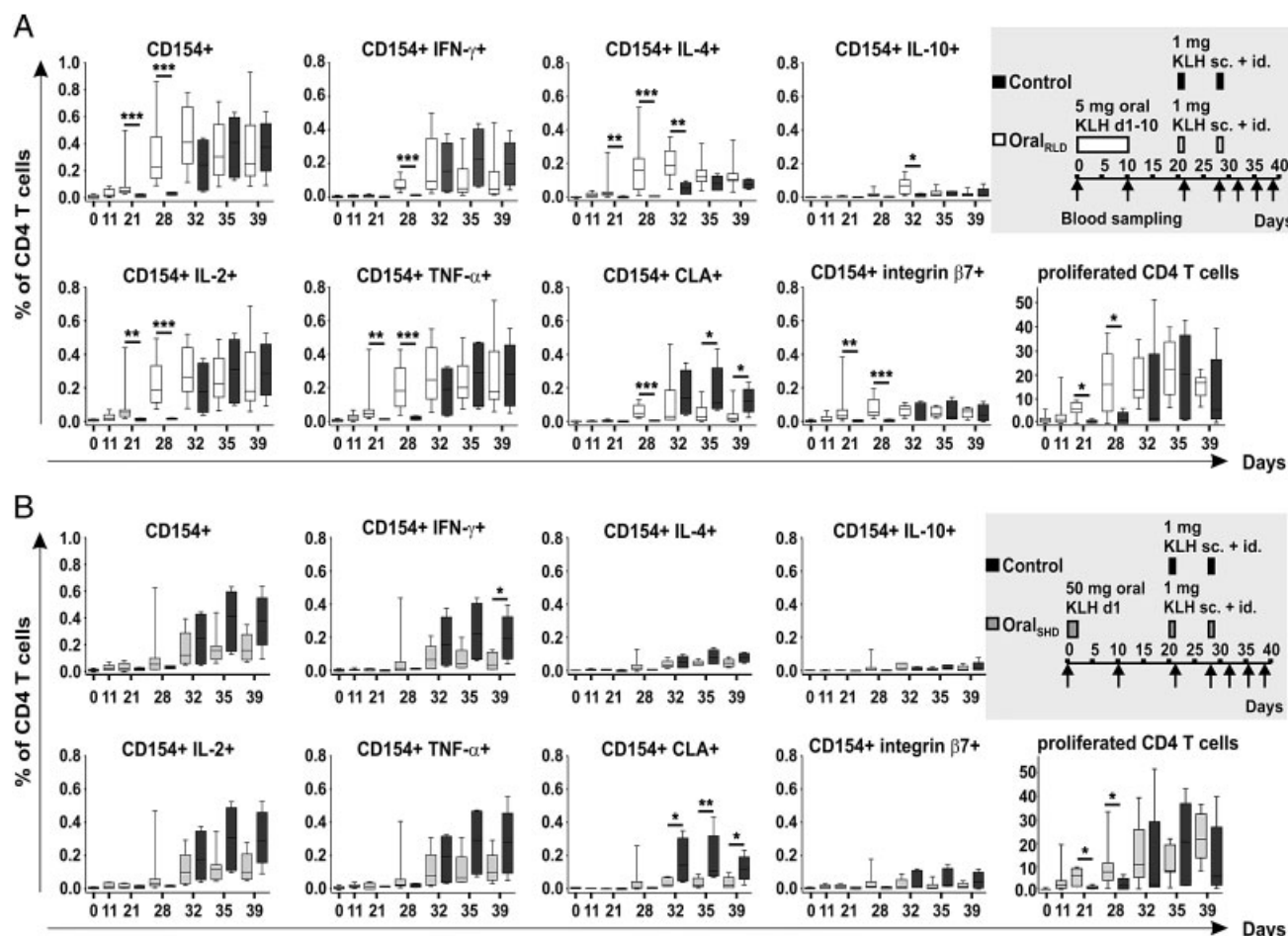


Figure 2. Effects of oral KLH on KLH-specific CD4⁺ T-cell responses induced by subsequent parenteral immunization. PBMC from the (A) oral_{RLD} group (*n* = 8) and (B) oral_{SHD} (*n* = 7) group were isolated at days 0, 11, 21, 28, 32, 35 and 39 and analyzed by flow cytometry for CD4⁺ T-cell proliferation and for the expression of cytokines and homing receptors in comparison to controls (*n* = 6). Shown are the percentages of KLH-specific CD154⁺ cells, the percentages of cells expressing the indicated cytokines and homing receptors together with CD154, as well as the percentage of proliferated cells within the CD4⁺ T-cell population (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, Mann-Whitney test).

for IL-10 expressing cells, which were not detected at all (Fig. 2).

A faster CD4⁺ T-cell response after parenteral immunization was observed in the oral_{RLD} group (Fig. 2A). As noted above, orally primed antigen-specific CD4⁺ T cells were already detectable at day 21, prior to the first parenteral immunization (Fig. 1A). In contrast to the control group, the frequency of KLH-specific CD4⁺ T cells in the oral_{RLD} group increased already after the first parenteral immunization, and reached a maximum 4 days after the second parenteral immunization at day 32 (Fig. 2A). Again, similar kinetics were seen for all subpopulations of KLH-specific CD4⁺ T cells except for IL-10-producers, which were detected only at day 32.

Although CD4⁺ T-cell proliferation at days 21 and 28 was increased compared with controls in the oral_{SHD} like in the oral_{RLD} group, a single oral dose of 50 mg KLH did not induce an antigen-specific CD4⁺ T-cell response up to day 21 (Fig. 1A) and did not significantly affect KLH-specific CD4⁺

T-cell kinetics (Fig. 2B). In comparison to the control group, the proportion of KLH-specific CLA⁺CD4⁺ T cells (d32–39) as well as the proportion of IFN-γ-producing CD4⁺ T cells (d39) were lower in the oral_{SHD} group only at later time points (Fig. 2B).

Oral KLH shifts functional subpopulations of specific CD4⁺ T cells induced by parenteral immunization

To investigate qualitative alterations of the parenterally induced systemic immune response by earlier oral KLH, we compared the cytokine pattern and homing receptor expression of KLH-specific CD4⁺ cells of the oral_{RLD}, oral_{SHD} and control group 4 days after the second parenteral immunization (d32, Fig. 3). At this time point, the frequencies of CD154⁺CD4⁺ T cells were similar in all groups and could be confirmed as KLH-specific by comparison with control cultures restimulated with anti-CD28 alone (Fig. 1B;

for subpopulations of CD154⁺CD4⁺ T cells, see Supporting Information Fig. 1C).

Compared with parenteral immunization only, repeated oral low-dose KLH shifted the proportion of cytokine producers within the KLH-specific CD4⁺ T cells toward more IL4⁺ and IL10⁺ and toward less IFN- γ ⁺, IL-2⁺ and TNF- α ⁺ cells (Fig. 3). In the oral_{SHD} group, the proportion of KLH-specific CD4⁺ T cells producing specific cytokines was generally intermediate between the proportions seen in the oral_{RLD} and control group. Only IL-4-producing KLH-specific CD4⁺ T cells were significantly less frequent in the oral_{SHD} compared with the oral_{RLD} group (Fig. 3). The frequency of KLH-specific CD4⁺ T cells positive for the skin-homing marker CLA was similarly reduced in the oral_{RLD} and oral_{SHD} group compared with controls. KLH-specific CD4⁺ T cells positive for integrin β 7 did not differ between the groups.

Oral KLH amplifies KLH-specific B-cell responses induced by subsequent parenteral immunization

To investigate effects of oral KLH on KLH-specific B-cell responses, we compared KLH-specific serum Ab in the oral_{RLD} group and control group (Fig. 4). Prior to the first KLH administration, whether oral or parenteral, we did not detect KLH-specific IgA and only minute amounts of KLH-reactive IgG (<41 AU/mL) (AU, arbitrary units) and IgM (<35 AU/mL) (Fig. 4A); KLH-specific IgE was not detected at all (data not shown). In the control group, KLH-specific IgA, IgG and IgM increased significantly after the second parenteral immunization (day 32) with maximal values at days 35 and 39. Oral KLH alone did not induce KLH-specific serum Ab (Fig. 4A, days 11 and 21). However, KLH-specific IgA and IgM increased earlier, *i.e.* after the first parenteral immunization (day 28), in the oral_{RLD} group compared with the control group (Fig. 4A). In addition to faster kinetics of IgA and IgM, the oral_{RLD} group showed higher KLH-

specific IgG and IgM serum Ab levels in the observed period compared with controls (Fig. 4A). KLH-specific B-cell responses were mostly unaltered by a single high dose of oral KLH (data not shown).

As shown above, low-dose oral KLH prior to parenteral immunization shifted the cytokine pattern of KLH-specific CD4⁺ T cells toward a Th type 2 response. Since the Ig subclass IgG4 seems to be associated with Th2 responses in humans [22, 23], we analyzed IgG subclasses of KLH-specific Ab. KLH-specific IgG1, IgG3 and IgG4 serum Ab appeared earlier and reached higher levels in the oral_{RLD} group compared with the control group (Fig. 4A). Furthermore, the IgG4/IgG3 ratio was significantly higher in the oral_{RLD} group compared with parenteral control group at day 39 (Fig. 4B) although values within groups varied considerably. KLH-specific IgG2 was detectable only in four of the eight subjects of the oral_{RLD} group (Fig. 4A).

Discussion

Oral uptake of antigens can lead to outcomes such diverse as systemic tolerance [16–18] and immunity [12–15] in humans. However, little is known about the underlying mechanisms and the function of Th cells induced by oral antigen. In this study, low-dose oral KLH induced an antigen-specific systemic CD4⁺ T-cell response with a shift toward a Th type 2 cytokine pattern. In a subsequent systemic immune response induced by parenteral immunization, low-dose oral KLH amplified B-cell responses and, most strikingly, shifted the cytokine pattern of KLH-specific CD4⁺ T cells from a Th type 1 toward a Th type 2, suggesting a novel effect of oral immunomodulation.

The frequencies of KLH-specific CD4⁺ T cells as identified by the expression of CD154 after short-term *in vitro* stimulation [20, 24] were low but similar to the frequencies of antigen-

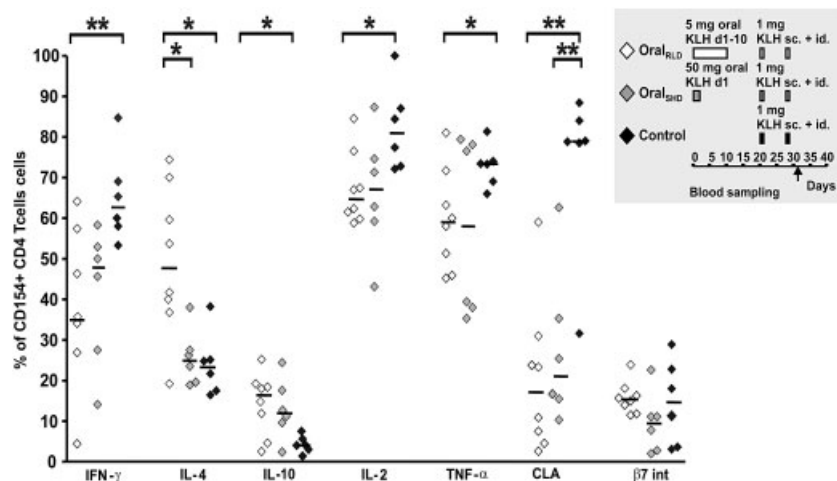


Figure 3. Oral KLH results in a shift of functional subpopulations of KLH-specific Th cells induced by subsequent parenteral immunization. PBMC were isolated at day 32 (4 days after the second parenteral immunization) and the expression of cytokines and homing receptors within the KLH-specific CD154⁺CD4⁺ T-cell population in the oral_{RLD} ($n = 8$), oral_{SHD} ($n = 7$) and control group ($n = 6$) was analyzed by flow cytometry. Individual data and medians are indicated (* $p < 0.05$, ** $p < 0.01$, Mann-Whitney test).

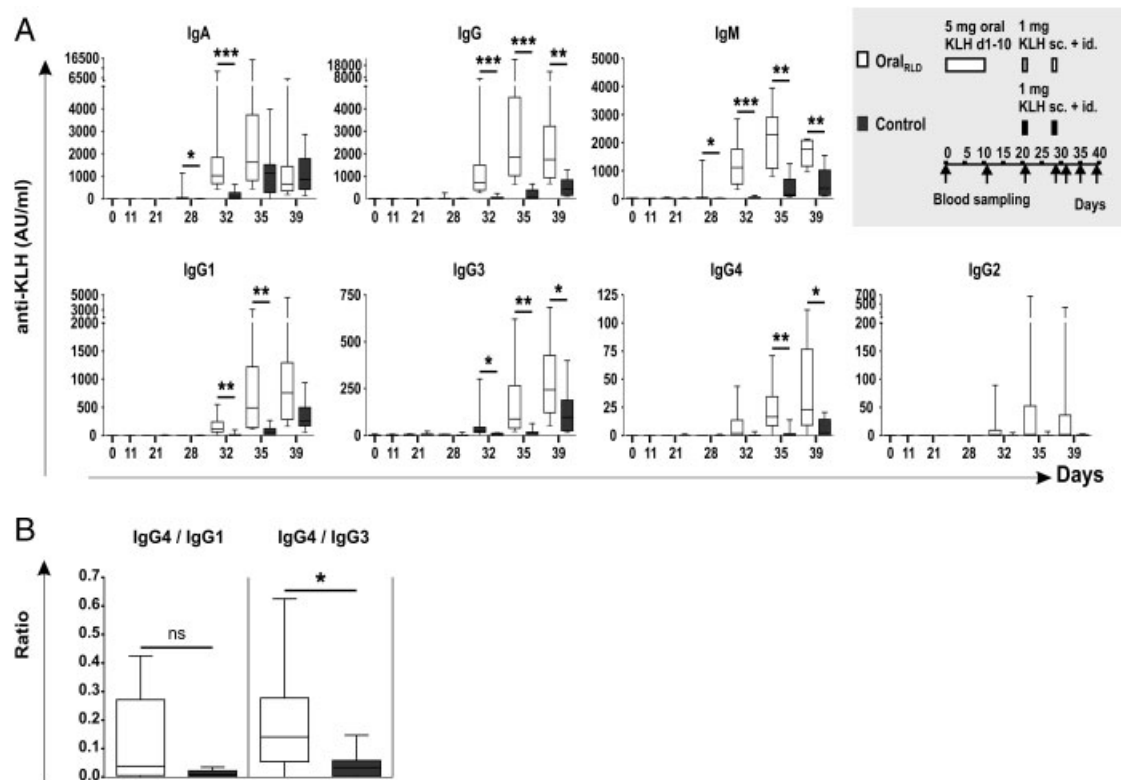


Figure 4. Oral KLH amplifies KLH-specific B-cell responses induced by subsequent parenteral immunization. Sera from subjects of the oral_{RLD} ($n = 8$) and control group ($n = 6$) were collected at days 0, 11, 21, 28, 32, 35 and 39 and analyzed for KLH-specific Ab as described in *Materials and methods*. (A) Kinetics of KLH-specific Ab responses. The amounts of KLH-specific Ab are denoted as arbitrary units (AU), because no specific anti-KLH control Ab are available. (B) Ratios of IgG subclasses IgG4/IgG1 and IgG4/IgG3 at day 39 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann-Whitney test).

specific Th cells observed in humans after vaccination [20, 21]. Furthermore, the CD4⁺ T-cell responses observed were clearly KLH specific as demonstrated by comparison with preimmune cultures or cultures stimulated by anti-CD28 alone.

It has been shown in mice that activation of T cells by DC imprints their tissue-specific homing potential. Accordingly, activation by DC from peripheral LN induces T cells with skin-selective homing potential [25–27]. On the contrary, T cells with gut-homing potential are induced by DC from Peyer's patches or MLN [25–29]. The selective capacity of murine intestinal DC to generate integrin $\alpha 4\beta 7$ CCR9⁺ gut-homing T cells involves retinoic acid [30] and seems to be restricted to the specialized subset of CD103⁺ MLN DC originating from the small intestinal lamina propria [31]. Similar mechanisms could explain the predominant expression of the skin-homing receptor CLA and the gut-homing receptor integrin $\beta 7$ on KLH-specific Th cells after parenteral and oral immunization, respectively, in our study and earlier human reports [13, 32–36].

Outcomes of oral uptake are antigen-dependent, e.g. bacterial toxins such as cholera toxins can be used as mucosal adjuvants [37, 38] to avoid tolerance induction, whereas cholera toxin B subunit lacking its toxic A fraction was found to augment tolerogenic effects [39]. In contrast to most food proteins, KLH is a very large protein with an established use as adjuvants [40] and, possibly, specific immunological effects after oral uptake. KLH is,

however, the only neoantigen with a history of clinical and experimental administration in humans, and an earlier human study found no differences in T- and B-cell responses after feeding KLH or ovalbumin [41].

Outcomes of oral antigen are also dose dependent: in mice, a single high dose (>0.5 mg/g body weight) of oral antigen suppresses most immune responses by direct T-cell inactivation [42], whereas multiple low doses (<0.1 mg/g body weight) of oral antigen favor the generation of regulatory cells [5, 43]. Oral antigen doses in our and other human studies [16, 17], however, have been one to two orders of magnitude lower, and parenteral immunization doses varied from 0.01 to 1 mg of KLH. In our study, repeated feeding with a low dose of KLH accelerated the Th-cell response after parenteral immunization, whereas earlier studies reported reduced DTH [17] and/or reduced proliferation of PBMC stimulated with KLH [16, 17], following KLH feeding in human volunteers. These studies used higher oral and lower parenteral KLH doses compared with our study which confirms a dose-dependent effect of oral KLH. Interestingly, low-dose oral KLH utilized in this study induced measurable systemic T-cell responses not seen in the earlier studies and effectively modulated systemic immune responses induced by parenteral KLH.

Induction of functional CD4⁺ T cells is also supported by KLH-specific B-cell responses which, in parallel to an earlier occurrence of KLH-specific Th cells, appeared faster and, as reported by others

[17], were even amplified after oral KLH. Th cells play a pivotal role in the development of antigen-specific B-cell responses against protein antigens [44, 45] and thus are probably required in the selection, expansion and differentiation of KLH-specific B cells.

Parenterally induced systemic immune responses were not merely amplified after oral KLH. The most striking effect of oral KLH was a shift in the cytokine pattern of KLH-specific CD4⁺ T cells from a Th type 1 toward a Th type 2, with more IL-4- and less IFN- γ -producing cells. A similar shift has been found in mice after intraperitoneal immunization with KLH, which reduced IFN- γ and increased IL-4-secreting cells induced by subsequent parenteral immunization not only for KLH, but also for the unrelated antigen guinea pig myelin [46]. KLH-specific IL-10-producing cells were only induced after oral priming with KLH and may represent Th cells, supporting the differentiation of B cells [47, 48]. Alternatively, these could be self-controlled Th1 cells, recently identified by the coexpression of IFN- γ and IL-10, which are believed to limit inflammatory responses [49–52].

It has been proposed that the special environment in mucosal tissues favors the induction of tolerance, the production of IgA Ab and also Th type 2 responses [2, 3]. Mucosal DC may be involved in the induction of Th type 2 responses, because it has been shown *in vitro* that murine Peyer's patches [53] and MLN DC [54] induce the differentiation of Th type 2 cells. Th type 2 responses have also been found after priming with low doses of antigen by nonmucosal routes in mice [55] and it could be speculated that compared with parenteral immunization lower amounts of antigen were available to the immune system after oral feeding in our study. However, we observed no priming of Th type 2 responses by parenteral low-dose KLH in our control, although the doses used were even lower than those in murine models. Therefore, we think that the priming of Th type 2 responses in our study is a specific effect of oral KLH.

Our protocol did not induce classical oral tolerance defined as specific suppression of cellular and/or humoral immune responses to an antigen by prior oral administration of the same antigen [1]. The decrease in CLA⁺ and IFN- γ -producing CD4⁺ T cells in the oral_{SHD} group could reflect tolerance of *e.g.* skin homing Th type 1 cells, and the reduction of Th type 1 cells in our study would be compatible with the induction of tolerized or regulatory T cells by oral KLH. Specific markers for antigen-specific regulatory T cells are not available so far, and the frequency of KLH-specific Th cells is very low; therefore, the identification by *e.g.* FOXP3 in combination with CD25 [56] is not sensitive enough for the quantification of regulatory T cells induced or expanded by our protocol. However, oral KLH even primed CD4⁺ T-cell proliferation and did not alter DTH reactions, providing evidence rather against the induction of regulatory T cells. If regulatory activity is involved in the shift toward Th type 2 cytokines, it should be abrogated by high-dose IL-2 or CTLA-4 blockade [2]. Because our blood samples were limited, this could not be assessed within the study reported here.

Alternatively, the observed cytokine shift toward a Th type 2 response after parenteral immunization might be mediated by pre-existing KLH-specific Th type 2 cells possibly primed in the MLN after oral KLH administration. MLN can act as crossover point

between mucosal and systemic immune systems, and therefore antigen-specific Th type 2 cells may migrate from MLN into peripheral LN [2]. Following subsequent parenteral immunization, antigen would be presented by DC not only to naïve T cells in peripheral LN [52], but also to pre-existing KLH-specific Th type 2 cells generated *via* the oral route. In this scenario, by rapid secretion of IL-4 these orally primed Th type 2 cells could induce Th type 2 cell differentiation in KLH-reactive naïve T cells [57].

To our knowledge, these are the first data to demonstrate that an oral antigen can modulate a subsequent systemic immune response in humans toward Th type 2 cytokine production and amplified B-cell responses. If oral antigen could likewise modulate established Th type 1 responses, then such a shift toward a Th type 2 response would allow the treatment of autoimmune and inflammatory diseases caused by antigen-specific pathogenic Th type 1 cells. The predominant Th type 2 response associated with high-titer serum Ab after oral priming could improve humoral immunization strategies.

Materials and methods

KLH

Clinical grade KLH (Immucothel[®]) was obtained from Biosyn (Fellbach, Germany). The production of acid-resistant capsules for oral administration was performed under GMP conditions by Rentschler Pharma (Laupheim, Germany).

Subjects and study design

A total of 21 healthy volunteers were recruited for the study. The repeated low-dose oral group (oral_{RLD}, three males, five females; mean age 33 years, range 23–49) ingested 5 mg KLH in acid-resistant capsules from day 1 to day 10 (Fig. 1). The single high-dose oral group (oral_{SHD}, four males, three females; mean age 38 years, range 27–53) ingested 50 mg KLH in acid-resistant capsules on day 1. All subjects were parenterally immunized in the upper arm with 0.5 mg KLH intradermally and 0.5 mg KLH subcutaneously on day 21 and day 28. In total, 50 mL of heparinized blood samples were taken as indicated in the respective time schedule of all figures. The parenteral control group (controls, three males, three females; mean age 28 years, range 26–30) was immunized only parenterally on days 21 and 28 as described. Blood samples were taken at regular intervals as shown in the figures. The study was approved by the local ethics committee and informed written consent was obtained from all the participants.

Cell isolation and stimulation

PBMC were obtained after density gradient centrifugation. Antigen-specific CD4⁺ T cells were identified through short-term

stimulation as described by Frentsch *et al.* [20]. Briefly, PBMC were stimulated with 200 µg/mL KLH and 1 µg/mL anti-CD28 (L293, BD Biosciences, Heidelberg, Germany) for 8 h in RPMI (PAA, Cölbe, Germany) supplemented with 10% fetal calf serum (Linaris, Wertheim, Germany), 4 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from PAA) at a concentration of 2×10^6 cells/mL. *Staphylococcus enterotoxin B* (7 µg/mL, Sigma-Aldrich, Taufkirchen, Germany) served as positive control. Negative controls contained culture medium and the costimulatory mAb anti-CD28. Brefeldin A (10 µg/mL, Sigma-Aldrich) was added for the last 4 h. At the end of incubation, EDTA (2 mM) was added for 15 min at room temperature. Cells were fixed in 2% formaldehyde in PBS for 20 min and washed in PBS/0.5%BSA/0.1%NaN₃.

Cell staining and flow cytometry

Cells were permeabilized in FACS Permeabilization Solution 2 (BD Biosciences) and stained for flow cytometry in PBS/0.5%BSA/0.1%NaN₃ containing 0.1% Beriglobin (ZLB Behring, Hattersheim, Germany). The following mAb were used: 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) and anti-integrin β7 (FIB504), all from BD Biosciences; anti-CD19 (HIB19), anti-CD14 (61D3) and anti-CD62P (AK-4), all from eBioscience (San Diego, CA, USA). Cells were analyzed on a FACSCalibur (BD Biosciences). In total, 100 000 CD4⁺ T cells were acquired. Data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA). KLH-specific Th cells were quantified as proportion of CD154⁺CD4⁺ cells after stimulation with anti-CD28 and KLH corrected for the proportion of CD154⁺CD4⁺ cells after stimulation with anti-CD28 alone (Supporting Information Fig. 1). According to the earlier studies [20, 24] a threefold increase above background was selected as cut-off point.

For proliferation analysis, the CellTrace™ CFSE Cell Proliferation kit (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions. Briefly, 4×10^6 cells/mL were stained with 5 µM CFSE for 10 min at 37°C and analyzed by flow cytometry as described above. In all groups analyzed, the proportion of proliferated CD4 T cell after stimulation with anti-CD28 alone was always below 3%.

Measurement of KLH-specific serum Ab

Flat-bottom polystyrene microtiter plates (Immunolon 2HB, VWR International, Darmstadt, Germany) were coated with KLH (10 µg/mL, Sigma-Aldrich) in PBS at 4°C overnight. After washing with PBS/0.05% Tween-20, plates were blocked with 1% BSA in PBS for 1 h at room temperature. Serial threefold dilutions of sera were prepared in supplemented RPMI and incubated for 2 h at 37°C. Biotinylated anti-IgA (G20-359), anti-IgM (G20-127), anti-IgG1 (G17-1), anti-IgG2 (G18-21), anti-IgG4 (JDC-14) and anti-IgE (G7-

26), all from BD Biosciences; anti-IgG (HP6017) and anti-IgG3 (HP6050), both from Sigma-Aldrich, were diluted in 1% BSA in PBS added for 2 h at room temperature. Plates were incubated with Streptavidin-horseradish peroxidase (Dianova, Hamburg, Germany) for 30 min, developed with 3,3',5,5'-Tetramethylbenzidine (Sigma-Aldrich) and stopped with an equal volume of 1 N H₂SO₄. The absorbance was determined at 450 nm on a microplate reader (Tecan, Crailsheim, Germany). A reference serum containing high-titer sera from immunized subjects was defined to contain 1000 AU of KLH-specific IgA, IgG and IgM. The amounts of IgG subclasses within the reference serum were determined using the total IgG standard curve.

Statistical analysis

Nonparametric statistical tests were applied. The Mann–Whitney test and the Wilcoxon matched pairs test were used to analyze the differences between unpaired and paired data, respectively. Statistical analyses of subpopulations were performed if KLH-specific CD4⁺ T cells were present. *p*-Values <0.05 were considered significant. Selected data are presented as box plots which show medians, 25th and 75th percentiles, and minimum and maximum values.

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Abbreviations: AU: arbitrary units · CLA: cutaneous lymphocyte antigen · DTH: delayed-type hypersensitivity · KLH: keyhole limpet hemocyanin · RLD: repeated low dose · SHD: single high dose

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Supporting Information

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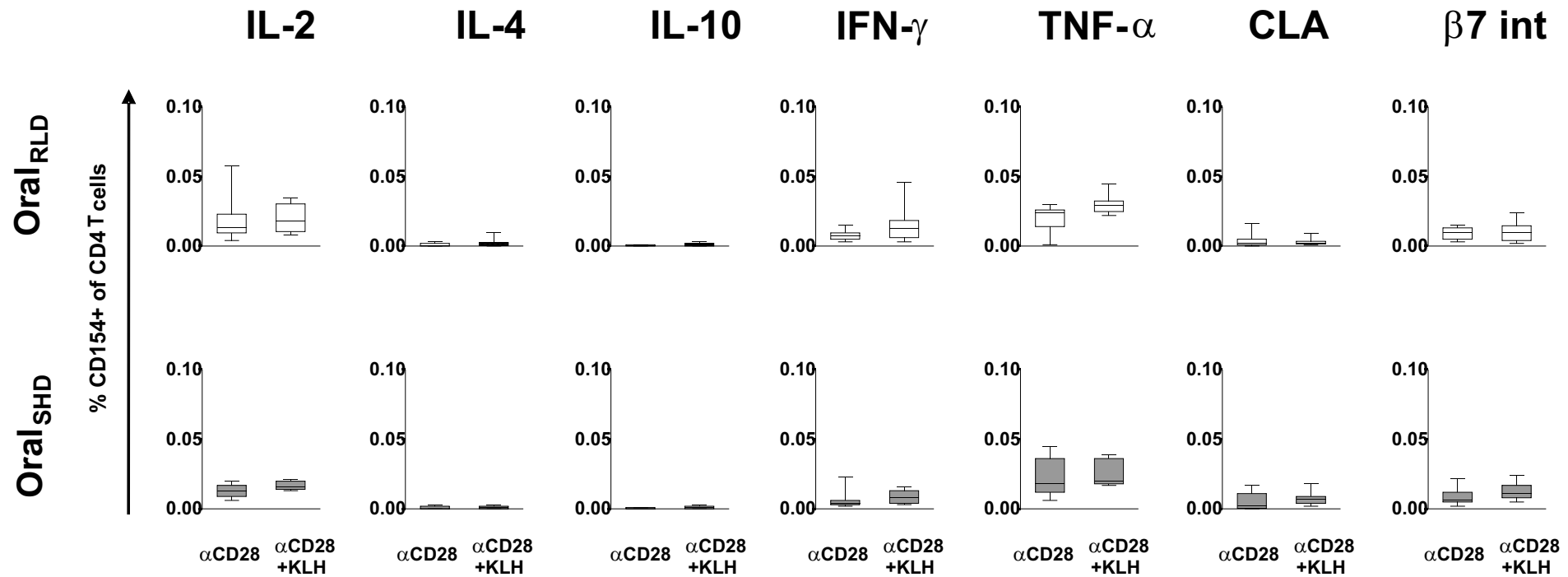
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Modulation of systemic antigen-specific immune responses by oral antigen in humans

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Supplemental Figure 1A

Day 0

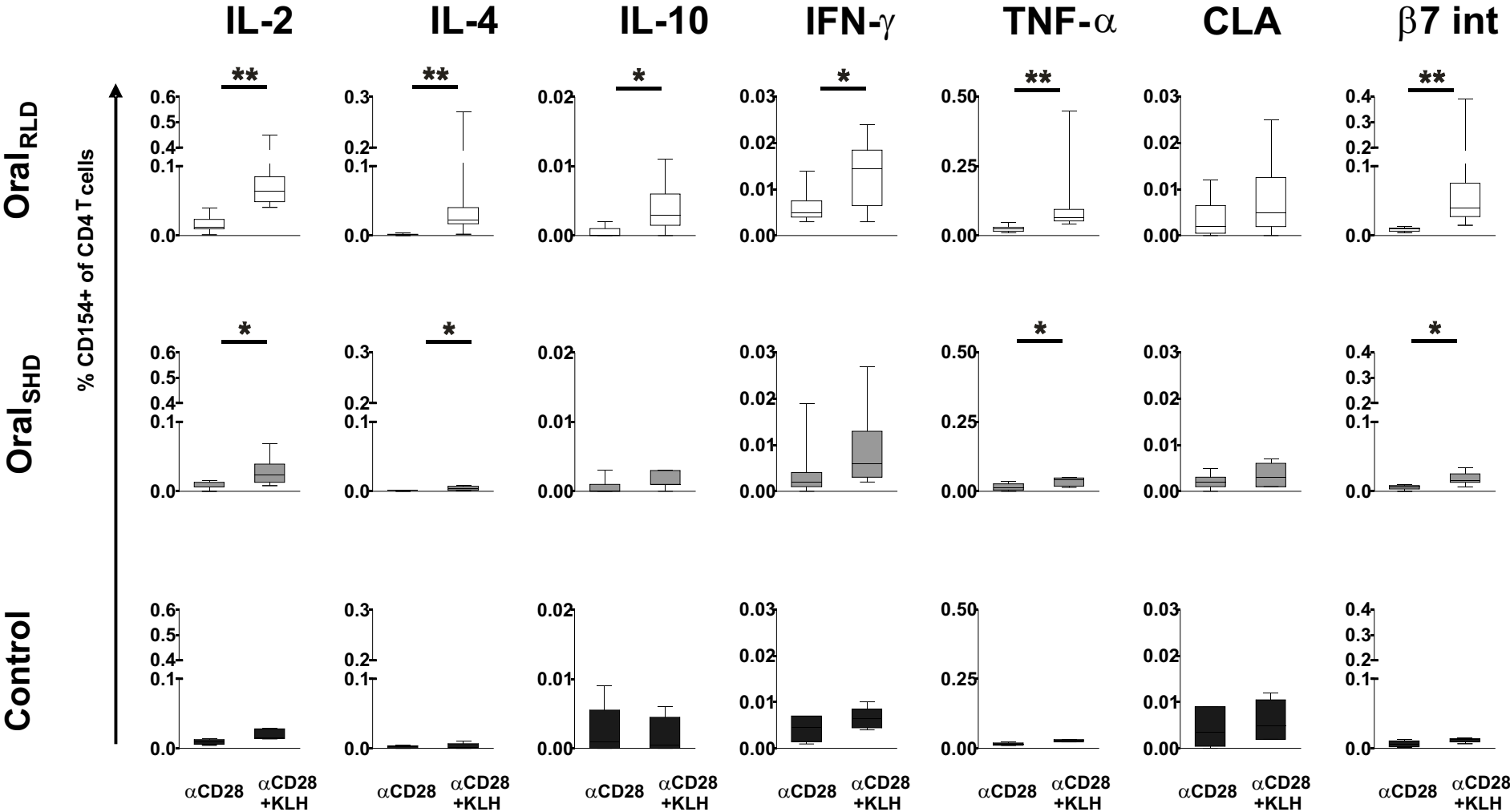
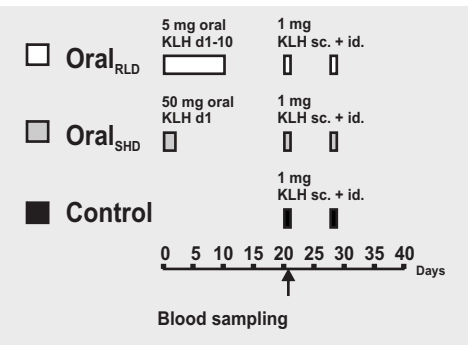


Supplemental Figure 1. Detection of KLH-specific CD4 T cells

PBMC were isolated at (A) day 0, (B) day 21, (C) day 32 and analyzed by flow cytometry in the oral_{RLD} (n=8), the oral_{SHD} (n=7) and the control group (n=6). The percentages of cells expressing the indicated cytokines and homing receptors together with CD154 within the CD4 T cell population were compared between cultures stimulated with anti-CD28 + KLH and cultures stimulated with anti-CD28 alone

Supplemental Figure 1B

Day 21



Supplemental Figure 1C

Day 32

