In vivo modulation of antigen-experienced cells in response to high-dose oral antigen: deletion but no evidence for alterations in the cytokine phenotype

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Abstract

Whether also antigen-experienced CD4⁺ T cell populations undergo modulations upon oral antigen uptake supporting systemic unresponsiveness is still not fully understood. Using an adoptive transfer model with chicken ovalbumin (OVA)-specific T cells, we demonstrated that absolute numbers of transferred ex vivo-isolated CD4⁺ memory T cells and in vitro-polarized T_h1 cells considerably decrease within spleen and liver upon repetitive OVA feeding. As a consequence, these mice did not mount a delayed-type hypersensitivity reaction after OVA challenge. OVA-specific T_h1 cells re-isolated from the liver showed augmented signs of apoptosis. However, there was no evidence that transferred effector or memory T cells acquired a regulatory phenotype, became anergic or underwent immune deviation. Our data suggest that oral antigen application does not induce alterations in the phenotype of CD4⁺ effector and memory T cells. Instead, deletion of antigen-experienced CD4⁺ T cells preferentially within the liver might be a major mechanism contributing to antigen-specific systemic unresponsiveness upon oral antigen uptake.

Introduction

The induction of tolerance as a therapeutic principle implies a permanent shift of an immune reaction from inflammation to antigen-specific unresponsiveness. Studies in T cell mediated rodent models of various diseases have suggested that tolerance induction might be effective to treat inflammations. Feeding the specific antigen did not only prevent experimental encephalomyelitis but also ameliorated an ongoing disease (1). Similar effects were shown in a colitis model (2), in murine graft-versus-host disease (3) and mild forms of asthma in mice (4). However, the effects on established immune reactions were disappointing weak and mechanisms of successful induction or failing of tolerance remained unclear. Specific tolerance was accompanied by down-regulation of effector cytokines and could be transferred by T cells (2). These findings suggest that the modulation of antigen-specific effector and memory T cells plays a key role in re-establishing systemic antigen-specific unresponsiveness. Adoptive transfer studies using chicken ovalbumin (OVA)-specific T cells, allowing to study modulations and fate of antigen-experienced CD4⁺ T cells on single-cell level, have shown that intravenous application of the antigen may lead to anergy (5) and deletion of T_h1 cells within the liver (6). In the present study, we investigated mechanisms imposed on single effector or memory CD4⁺ T cell upon oral antigen uptake using adoptive transfer models of OVA-specific CD4⁺ memory and T_h1 cells.

Methods

Antibodies

A digoxigenin-conjugated clonotypic antibody (KJ1.26) specific for a T cell receptor recognizing the OVA-derived peptide OVA323–339 in the I-A<sup>d</sup> context in DO11.10 mice, anti-IFN-γ-FITC (XMG6), anti-IL-4 (11B11) and anti-Fcγ receptor II/III (2.4G2) were kindly provided by the Deutsches Rheumafor- schungszentrum (Berlin, Germany). Labeled Annexin V and other antibodies were from BD Biosciences (Heidelberg, Germany). Rat IgG was from Sigma (Deisenhofen, Germany).

Reagents

Metrizamide was obtained from Axis Shields (Oslo, Norway). The OVA-derived peptide OVA323–339 (H-ISQAVHAAHAEINEAGR-OH) was synthesized at the Biochemical Institute of

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The Humboldt-Universität (Berlin, Germany). IFNγ and IL-12 were from BD Biosciences. All other reagents and cell culture supplements were purchased from Sigma and were of the highest purity available.

**Cell lines**

The hybridoma cell lines 53-6.72 (CD8), M170 (Mac-1), 2.4G2 (Fcγ receptor II/III) and MEL-14 (CD62L) were obtained from the American Type Culture Collection (Manassas, VA, USA) and were used to prepare antibody-containing supernatants for panning. The murine B-cell line A20 for antigen presentation was kindly supplied by E. Schmitt (Mainz, Germany).

**Mice**

DO11.10 mice expressing a T cell receptor, specific for OVA<sub>323-339</sub> presented in the I-A<sup>d</sup> context (7), and BALB/c mice were obtained from the Bundesinstitut für Risikobewertung (Berlin, Germany) at 8–10 weeks of age. All animals received human care according to the criteria outlined in the ‘Guide for the care and use of laboratory animals’ published by the National Institutes of Health (Bethesda, MD, USA).

**Ex vivo enrichment of CD4<sup>+</sup>CD45RB<sup>low</sup> memory T cell and naive CD4<sup>+</sup> T cells and in vitro polarization of OVA-specific T<sub>H</sub><sup>1</sup> cells**

CD4<sup>+</sup>CD45RB<sup>low</sup> T cells were enriched from spleens of older DO11.10 mice by panning using an antibody mixture including anti-CD8, anti-Mac-1, anti-Fcγ receptor II/III and anti-CD62L. Resulting preparations contained 50.0 ± 17.0% CD4<sup>+</sup>CD45RB<sup>low</sup> cells. Naive CD4<sup>+</sup> T cells containing ~98% CD4<sup>+</sup>CD62L<sup>high</sup>/CD45RB<sup>high</sup> cells were purified by MACS as previously described (8).

To obtain T<sub>H</sub><sup>1</sup>-polarized cells, total lymph node cells and spleen cells of naive DO11.10 mice were cultured in vitro (ratio 1:2) with 5 μg ml<sup>−1</sup> OVA<sub>323-339</sub>, 5 μg ml<sup>−1</sup> IL-4-depleting antibody, 10 ng ml<sup>−1</sup> IL-12 and 20 ng ml<sup>−1</sup> IFNγ according to the established methods (6, 8). After 6 days, cells were harvested, passed over a density gradient to remove dead cells and transferred into BALB/c mice.

**Oral antigen application model and re-isolation of donor cells**

*Ex vivo*-isolated CD4<sup>+</sup> memory T cells (5–7 × 10<sup>6</sup> CD45RB<sup>low</sup>/CD4<sup>+</sup>KJ1.26<sup>+</sup> cells), *ex vivo*-isolated CD4<sup>+</sup> naive T cells (2.5–5 × 10<sup>6</sup> CD62L<sup>high</sup>/CD4<sup>+</sup>KJ1.26<sup>+</sup> cells) or *in vitro*-polarized OVA-specific T<sub>H</sub><sup>1</sup> cells (1 × 10<sup>7</sup> per mouse) were injected into the tail vein of syngeneic BALB/c mice. Starting at day 1 after transfer, mice were fed five times every other day with 20 mg OVA in PBS or with PBS alone for control. Eventually, mice were sacrificed at day 11 and donor cells analyzed for expression of surface markers and intra-cytoplasmic cytokine expression (Supplementary Figure 1, available at International Immunology Online).

Lymphocytes were isolated from *in situ*-rinsed livers by metrizamide density gradient centrifugation as previously described (6). In brief, murine livers were perfused *in situ* with 2 ml pre-warmed digestion medium containing 5% FCS, 2 mg ml<sup>−1</sup> collagenase IV and 0.2 mg ml<sup>−1</sup> Dnase I injected into the portal vein. Organs were removed, cut into small pieces and digested in the above mixture (25 min, 37°C, constant shaking with 250 r.p.m.). Single-cell suspensions passed through a sieve were subjected to a one-step density gradient centrifugation with 26% metrizamide. Total lymphocyte numbers from single livers were 0.5–1.5 × 10<sup>7</sup>. Spleens or pooled lymph nodes were disrupted and passed through a fine mesh, yielding 1–1.5 × 10<sup>9</sup> and 3–7 × 10<sup>7</sup> lymphoid cells, respectively.

**Analysis of surface marker and intra-cytoplasmic cytokine expression by flow cytometry**

Single-cell suspensions from organs of recipient mice were prepared as described above. Non-specific antibody binding was blocked by pre-incubating the cells with 10 μg ml<sup>−1</sup> rat IgG and anti-Fcγ receptor II/III. To identify donor cells, the clonotypic antibody (clone KJ1.26) was used in combination with anti-CD4 and antibodies to various other surface markers. Absolute donor cell numbers were calculated by multiplying the frequency of CD4<sup>+</sup>KJ1.26<sup>+</sup> donor cells by the absolute cell numbers re-isolated from the respective organ. Memory cells were identified by CD45RB staining. Apoptosis was determined by Annexin V binding.

For *in vitro*-polarized cytokine staining, cells from single-cell suspensions were re-stimulated with 10 ng ml<sup>−1</sup> phorbol myristate acetate (PMA) and 500 ng ml<sup>−1</sup> ionomycin, and brefeldin A was added after 45 min. For antigen-specific re-stimulation, cells were incubated for 2 h with 5 μg ml<sup>−1</sup> OVA<sub>323-339</sub> presented by autologous antigen-presenting cells or by the B-cell line A20 before the addition of brefeldin A. After 5 or 6 h of total re-stimulation time, cells were harvested, stained for surface markers as described above and fixed with 2% paraformaldehyde. Cell membranes were permeabilized using a buffer consisting of 0.5% saponin and 0.2% BSA in PBS. Again, non-specific antibody binding was blocked with rat IgG and anti-Fcγ receptor II/III. Cells were double-stained for *in vitro*-cytoplasmic IFNγ, IL-4 and IL-10, respectively, and analyzed for donor-derived cells by flow cytometry. Stained cells were analyzed by four-color flow cytometry using a FACS Calibur and the CellQuest Software (BD Biosciences). Lymphocytes defined by low forward/side-scatter characteristics were gated for KJ1.26<sup>+</sup>CD4<sup>+</sup> cells. Depending on the donor cell frequency in the total, a total number of 2.5 × 10<sup>5</sup> and 1.5 × 10<sup>4</sup> to obtain 2 × 10<sup>8</sup>–1 × 10<sup>7</sup> donor-derived cells in the life-gate as defined above.

**Induction of a delayed-type hypersensitivity reaction**

A delayed-type hypersensitivity (DTH) reaction was induced using a modified version of published protocols (9, 10). In brief, mice adoptively transferred and treated as described above received one subcutaneous injection of 10 μg OVA in 10 μl PBS into the right hind footpad. For control, 10 μg BSA in 10 μl PBS were injected into the left hind footpad of the same mice. Twenty-four hours after challenge, footpad thickness was measured and some animals were subsequently sacrificed for further analysis.

**Data analysis**

For statistical analysis, P-values were determined by Mann–Whitney test using the Minitab Statistical Software for Macintosh (Minitab, State College, PA, USA).
Results

OVA feeding induces a decrease of antigen-specific CD4+ effector and memory T cells but no alteration in their cytokine phenotype.

OVA-specific CD4+CD45RBlow memory cells enriched ex vivo were adoptively transferred and mice subsequently fed with OVA. Independent of the antigen administration, peripheral and mesenteric lymph nodes as well as Peyer’s patches and gut contained very few donor cells (data not shown), whereas considerable numbers were found in spleen and liver. In mice that had received OVA, absolute numbers of donor cells declined within both organs compared with control mice (Fig. 1). Also, adoptively transferred naive OVA-specific CD4+ T cells became fewer within spleen, liver and lymph nodes after repetitive OVA feeding (Fig. 2).

Whereas 17.3 ± 5.5% of memory cells produced IFNγ, only 2.1 ± 0.7% were IL-10+ and 1.2 ± 0.4% IL-4+. Repetitive OVA feeding did not affect the frequencies of IFNγ+ cells among donor cells. The percentages of IL-10+ cells were slightly increased within the spleen. As a consequence of the decrease in total CD4+CD45RBlow donor cell numbers, absolute numbers of IFNγ+ donor cells tend to be reduced and total cell numbers of IL-10+ cells were not altered compared with the control mice (Fig. 3). IL-4+ donor cells remained low (data not shown).

Th1 effector cells polarized in vitro were preferentially found within spleen, liver and peripheral lymph nodes. Independent of the antigen administration, mesenteric lymph nodes as well as Peyer’s patches and gut contained negligible numbers of donor cells (data not shown). In PBS-fed control mice, adoptively transferred in vitro-polarized Th1 cells persisted in spleens and livers, whereas in OVA-fed mice their absolute numbers were significantly reduced (Fig. 4). In lymph nodes, persisting donor cells were reduced from 1–1.5 × 10⁶ to 1–2 × 10⁵. A single dose of 20 mg OVA fed at day 1 resulted in a decrease of absolute donor cell numbers only within the liver but not within the spleen at day 11 (Fig. 4).

Before injection, the OVA-specific Th1 cell population contained 58.6 ± 9.0% IFNγ+ and 1.8 ± 0.3% IL-10+ cells. The fraction of IL-4+ cells was <0.1%. As observed with ex vivo-isolated memory cells, OVA feeding induced no alterations in the frequencies of IFNγ+ cells among donor Th1 cells within the spleen and only a slight decrease within the liver. Due to the loss in total donor cells, absolute numbers of IFNγ+ donor cells were significantly reduced (Fig. 5). The frequencies of IL-10+ and IL-4+ donor cells were very low (Fig. 6).

To exclude unresponsiveness on the level of T cell receptor triggering that could have been circumvented by the non-specific PMA/ionomycin standard re-stimulation prior to intra-cyttoplasmic cytokine staining, cells re-isolated from spleen were re-stimulated with the specific antigen OVA₃₂₃–₃₃₉. Since ex vivo spleen cell preparations contained sufficient numbers of autologous antigen-presenting cells, no further antigen-presenting cells were added. Irrespective of the initial in vivo treatment, frequencies of OVA-specific IFNγ+ cells were significantly lower upon antigen-specific re-stimulation in vitro, compared with the standard conditions using PMA/ionomycin. This observation was independent of the type of antigen-presenting cell as determined using the B-cell line A20 (data not shown). The IFNγ-producing subfraction among OVA-specific Th1 cells re-isolated from mice that were fed with OVA was not significantly reduced compared with control mice arguing against anergy of proximal T cell...
receptor signaling levels (Fig. 6A). The same applied to donor-derived cells re-isolated from the liver (OVA fed 40.2 \pm 2.8\%, control 31.0 \pm 4.2\%) after re-stimulation with OVA 323–339 presented by A20 cells. Furthermore, we did not observe a switch in the cytokine phenotype of donor Th1 cells re-isolated from spleen (Fig. 6B) or liver (data not shown) toward IL-4 or IL-10 production upon OVA feeding.

In vivo remaining OVA-specific Th1 cells do not express surface markers of regulatory cells but display increased signs of apoptosis within the liver

To investigate further potential phenotypic in vivo modulations on single donor cell level, OVA-specific Th1 cells were stained for CD25 and αE-integrin. As a consequence of in vitro culture, before injection, \sim98\% of the Th1 cells expressed CD25 primarily as an activation marker. Irrespective of the in vivo treatment, 11 days after transfer, only minorities of donor cells were CD25\+. The frequencies of αE+ cells with 1.3 \pm 0.1\% prior to transfer remained low within donor cells and were not affected by oral OVA application (Table 1).
Checking site-specific apoptosis should shed some light on mechanisms for the decrease in absolute numbers of donor cells. Prior to transfer, 1.7 ± 0.6% of cells from the Th1-polarizing cultures bound Annexin V. The proportions of apoptotic OVA-specific Th1 cells in control mice were ~10–20% over time in spleen and liver. Compared with these controls, single OVA feeding slightly increased the proportion of OVA-specific Th1 cells that underwent programmed cell death. Repetitive oral antigen application induced a small increase in the percentage of Annexin V+ donor cells within the spleen at day 11. Within the liver, repetitive antigen feeding led to significantly higher rates of apoptosis among OVA-specific Th1 cells compared with the respective control mice that did not receive the antigen (Fig. 7).

Adoptively transferred and OVA-fed mice do not mount a DTH reaction in response to OVA challenge

Functional consequences of oral antigen treatment in vivo were demonstrated by local DTH reactions. OVA feeding after adoptive transfer of OVA-specific Th1 cells resulted in significantly lower footpad swelling compared with control mice (Fig. 8). Percentages of donor cells within draining lymph nodes were higher in control mice as compared with OVA-fed mice. Furthermore, OVA injection into the footpad induced an increase in the frequencies of OVA-specific Th1 cells in control but not in OVA-fed mice (Table 2).

To determine whether low cell numbers might be responsible for lacking DTH reaction, injected OVA-specific cells were titrated from $1 \times 10^6$ to $1 \times 10^7$ per mouse. Low numbers of injected OVA-specific Th1 cells, leading to comparable donor cells frequencies within the lymph nodes as after feeding (Table 2), were unable to promote a DTH reaction. Concomitantly, frequencies of OVA-specific cells did not increase within the lymph nodes draining the OVA-treated footpad as observed in fed mice. In contrast, a DTH reaction was accompanied by increasing percentages of OVA-specific cells within the draining lymph nodes (Table 3).

### Discussion

In the present study, we focused on the in vivo fate of antigen-experienced CD4+ T cells in response to OVA feeding. T cell receptor transgenic mice cannot be primed normally (11). Antigen persistence in immunized mice may interfere with the in vivo effects of feeding and with the in vitro phenotypic analysis of cells (11, 12). Therefore, we used an adoptive transfer model with OVA-specific ex vivo-isolated CD4+CD45RBlow memory T cells and in vitro-polarized Th1

### Table 1. Expression of CD25 and αE by transferred Th1 cells in spleen and liver

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<tr>
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<th>CD25 (%)</th>
<th>αE (%)</th>
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<tr>
<td></td>
<td>Control</td>
<td>5 × OVA p.o.</td>
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<tr>
<td>Spleen</td>
<td>4.7 ± 2.4</td>
<td>7.2 ± 5.7</td>
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<tr>
<td>Liver</td>
<td>0.5 ± 0.2</td>
<td>1.0 ± 0.5</td>
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Total spleen and liver cells were obtained from BALB/c mice transferred with $1 \times 10^7$ in vitro-polarized Th1 cells and fed with OVA and PBS for control, respectively. Surface expression of CD25 and αE within donor cells was determined by flow cytometry. CD25 expression within Th1 cells before transfer was ~98%, αE expression 1.3 ± 0.1%. Mean values ± SDs of frequencies within donor-derived cells from two independent experiments with 5–11 mice.
cells that can be traced by a clonotypic antibody (13), thus allowing to study modulations on single donor cell level. In previous studies addressing oral tolerance in the presence of antigen-experienced cells, wild-type BALB/c mice were fed $10^3$–$10^4$ mg (14) or $5 \times 10^3$–$25$ mg OVA (12) following priming. On this basis, we decided to apply $5 \times 10^3$–$20$ mg OVA in our transfer system with CD4$^+$ effector and memory T cells (Supplementary Figure 1, available at International Immunology Online). This feeding regimen resulted in a deletion of adoptively transferred naive OVA-specific CD4$^+$ T cells without induction of cytokine expression.

For naive CD4$^+$ OVA-specific T cells, intravenous or oral OVA application has been shown to be accompanied by (i) phenotypic changes on single-cell level such as anergy (15) and (ii) induction of regulatory T cells and Th2 cells especially within the liver (16, 17) and deletion by apoptosis (13, 17). A slight time-dependent decrease observed in the IFN$\gamma$+ subfraction within adoptively CD4$^+$ effector T cells was largely independent of the presence of the antigen (6, 18) and the cells did not switch their cytokine phenotype in terms of an immune deviation. Arguing against T cell anergy, re-isolated cells were still able to synthesize IL-2 (data not shown) and IFN$\gamma$ in response to the specific antigen. In line with data on intravenously applied OVA peptide in animals...
adoptively transferred with antigen-experienced T cells (5, 11) or on oral OVA administration in primed mice (12, 14, 19), our in vivo observations on effector and memory T cells after oral OVA application support the concept that antigen-experienced T cells might be irreversibly committed due to an epigenetic imprinting during polarization (8, 20, 21).

OVA feeding did not support the expression of typical markers for regulatory T cells such as FoxP3 (data not shown), CD25 or $\alpha E$ in the $T_{h1}$ cells remaining in vivo. Thus, our observations did not furnish evidence for the induction of regulatory T cells from antigen-experienced cells upon antigen feeding. CD62L$^{hi}$CD45RB$^{hi}$ OVA-specific CD4$^+$ T cells became also fewer within secondary lymphoid organs and the liver after repetitive OVA feeding, naive CD4$^+$ T cells acquired a CD25$^+$FoxP3$^+$ phenotype (C. Siewert, personal communication). Therefore, under these conditions, differentiation into regulatory T cells might rather be expected for naive CD4$^+$ T cells (16, 22).

Antigen-experienced cells displayed increased frequencies of cells undergoing apoptosis in response to OVA feeding and frequencies of remaining donor cells were too low to support a DTH reaction. These findings suggest that deletion by apoptosis might be a major mechanism contributing to tolerance and additional mechanisms, albeit not fully excluded, do not necessarily have to contribute to systemic unresponsiveness. Recently activated $T_{h1}$ cells might be prone to programmed cell death. Deletion after an immunogenic feeding protocol using cholera toxin (Supplementary Figure 2, available at International Immunology Online) suggested that apoptosis might also be activation induced (23) although the immunogenic function of mucosal adjuvant established for naive cells (24) might not be directly transferable to antigen-experienced cells (25). Furthermore, ex vivo-isolated CD45RB$^{lo}$ memory cells and naive CD4$^+$ T cells displaying a more resting ‘natural’ phenotype were also reduced upon feeding. However, deletion has been observed in various models using naive or antigen-experienced cells (6, 13, 26). Therefore, deletion of $T_{h1}$ cells seems to be rather a ubiquitous mechanisms than specific for oral tolerance or antigen-experienced cells.

After 24 h, ~80–90% of intravenously injected $T_{h1}$ effector cells and CD4$^+$CD45RB$^{lo}$ memory T cells are found within spleen and liver (27–30). This distribution was not altered after OVA feeding and very few donor cells were detectable e.g. within mesenteric lymph nodes or gut (data not shown). Donor cell apoptosis was preferentially found within the liver, suggesting a special role of this organ in effector and memory T cell modulation. In this respect, our data confirm previous findings showing that naive OVA-specific CD4$^+$ T cells become deleted within the liver upon repetitive high-dose OVA feeding (17). In our model using BALB/c mice that ubiquitously present the antigen, we cannot formally exclude that T cells accumulate by selective trapping within the liver after being modulated elsewhere (26, 31), and, thus, the liver functions simply as a graveyard for dying cells (32).

Models with liver-restricted antigen presentation are required to prove antigen presentation within the liver essential for in vivo modulation of $T_{h1}$ cells. However, the assumption that the liver is causal is considerably enforced by the observation that oral antigen is presented within the liver and that CD11c$^+$ hepatic dendritic cells induce in vitro and in vivo apoptosis in antigen-specific naive CD4$^+$ T cells by decrease of IL-12 (17).

The conditions for oral tolerance induction in the presence of effector or memory T cells are still under discussion. In a model with intra-peritoneal priming using 20 μg OVA in complete Freund’s adjuvant, tolerance, as demonstrated by reduced OVA-specific antibody levels, was only inducible at early time points and after multiple feedings, whereas memory cells were speculated to become refractory (14). However, lacking tolerance in this system at later time points might be due to differences in humoral and the cellular immune responses (19), since in our model numbers of ex vivo-isolated memory T cells decreased after feeding. In polyclonal BALB/c mice primed by subcutaneous injection of 100 μg OVA in complete Freund’s adjuvant, feeding of 2–200 mg OVA up to 7 days after immunization produced a dose-dependent suppression of an OVA-specific DTH reaction (12, 19). Using the adoptive transfer model, we confirmed this finding and suggest deletion of $T_{h1}$ cells by apoptosis focused within the liver to be a major mechanism.

**Supplementary data**

Supplementary Figures 1 and 2 are available at International Immunology Online.

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**Abbreviations**

DTH delayed-type hypersensitivity

OVA ovalbumin

PMA phorbol myristate acetate

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