Original article

New cohorts of naive T cells exacerbate ongoing allergy but can be suppressed by regulatory T cells

Although as pretreatment oral tolerance is a potent means to achieve systemic suppression, its application in ongoing disease is controversial. Here we propose that availability of naive T cells may critically determine whether immunological tolerance is achieved during ongoing antigenic reactivity. Infusion of naive antigen-specific T cells into mice directly prior to eliciting a secondary T_H2 response induces these naive cells to actively engage in the antigenic response despite presence of established memory. Naive antigen-specific T-cells divided faster, produced more interleukin (IL)-2, IL-4 and IL-5 and enhanced immunoglobulin E (IgE) release during a secondary T_H2 response, compared with naive T cells that were infused prior to a primary response. Despite such contribution by new cohorts of naive T cells co-infusion of mucosal Tr together with naive T cells could suppress enhanced IgE release during a secondary T_H2 response. We conclude that naive T cells contribute to a secondary T_H2 response and although they can still be suppressed in the presence of sufficient numbers of mucosal Tr, they may interfere with potential therapeutic application of mucosal tolerance.

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Mucosal tolerance is a reliable mechanism to prevent potentially harmful systemic immune responses to innocuous food antigens and was therefore proposed as a putative therapy for a variety of inflammatory diseases ranging from auto-immunity to transplant rejection and allergy (1, 2). However, when translation from animal models to human disease proved to be extremely complex, oral tolerance became controversial. Despite its complexity, it is unequivocal that oral tolerance can be successfully achieved in humans after antigen feed (3, 4). Antigen feed suppressed *in-vitro* recall responses, but failed to down regulate titers of antigen-specific immunoglobulin G (IgG), IgM and secretory IgA suggesting suppression differentially affects T and B-cell responses (3). Intriguingly, this does not seem to apply to IgE mediated allergy

as it was recently shown that desensitization by oral immunotherapy was highly successful (5).

Using murine models it was shown decades ago that repeated feeding induces active suppression of the IgE response by development of a population of suppressor cells in the Peyer's patches (PP) of the gut-draining lymphoid tissue (6). Recent experiments confirmed and extended the finding of these suppressor cells, now named regulatory T cells (Tr) which differentiate from naive T cells in PP as well as mesenteric lymph nodes (MLN) and can be found in both $CD25^+$ and $CD25^- CD4^+$ T-cell populations (7). Moreover, patients that have outgrown cow's milk allergy show a similar allergen-responsive $CD4^+ CD25^+$ regulatory cell population in their peripheral blood (8). A caveat in the design of many murine models for suppression of food allergy is that oral tolerance is induced prior to antigen encounter (primary response) rather than during established disease

Abbreviations: MLN, mesenteric lymph nodes; OVA, ovalbumin; PP, Peyer's Patch; Tr, regulatory T cells.

(secondary response). Therefore, to understand the mechanism underlying tolerance induction in clinical studies this phenomenon needs to be studied in models of ongoing allergy. Previously, we have shown that antigen feed prior to a secondary T_H2 response could effectively prevent an increase in OVA-specific IgE response and was regulated by $CD4^+$ Tr (9).

We hypothesized that suppression of such ongoing allergy may not merely consist of inhibition of memory T-cell activation. Both naive and memory T cells may actively respond to second antigen encounter as has been shown for $CD8^+$ T cells in a secondary response to *Listeria monocytogenes* (10). Whether naive $CD4^+$ T cells also actively participate in a secondary immune response has been speculated on but not investigated (11).

We, therefore, wished to determine whether naive $CD4^+$ T cells are active participants in a secondary immune response and whether such participation may be a determining factor for the induction of Tr and ensuing tolerance during established memory T_H2 responses.

Materials and methods

Mice

BALB/c mice, aged 8–10 weeks, were purchased from Charles River (Maastricht, The Netherlands). DO11.10 transgenic (Tg) mice, which have a Tg T-cell receptor (TCR) specific for the ovalbumin (OVA) 323–339 peptide were bred and used at 8–12 weeks of age. Experiments were approved by the animal experimental committee of the VU medical center.

Antibodies

The anti-clonotypic antibody for the DO11.10 Tg TCR (KJ1.26) was biotinylated, according to manufacturer's protocol (Molecular Probes, Leiden, The Netherlands). PE conjugated anti-CD4 (GK1.5), anti-CD25 (PC61), anti-CD45RB (16A), anti-CD69 (H1.2F3), APC-conjugated anti-CD62L (MEL14), anti-CD25 (PC61), streptavidin-APC and PerCP conjugated-streptavidin were obtained from BD-Pharmingen (Woerden, The Netherlands).

Adoptive transfer and CFSE labeling of donor cells

To obtain mice with a number of naive antigen-specific T-cells that is sufficient for following their fate after *in vivo* stimulation, the method of Kearny (12) was used with minor modifications as

described previously (7, 13). Briefly, lymph nodes and spleens were isolated from DO11.10 mice and single cell suspensions were enriched for $CD4$ cells using negative depletion of contaminating cells with dynal beads (Dynal GmbH, Hamburg, Germany) (7, 13). Enriched $CD4^+$ T-cells, were labeled with 5,6-carboxy-succinimidyl-fluoresceine-ester (CFSE) (Molecular Probes, Leiden, The Netherlands) (7). Each mouse received 1.10^7 $CD4^+$ KJ1.26 $^+$ cells by intravenous (i.v.) injection. Flow cytometry (FACS Calibur; BD-Pharmingen) of the transferred cells showed that 98% expressed high levels of CD45RB, indicating they had a naive phenotype.

Tolerance induction and antigenic stimulation

BALB/c mice were fed (i.g.) 25 mg of OVA grade V (Sigma Aldrich, Zwijndrecht, The Netherlands) on day -7 (Table 1, group B), while control 'T_{H2}' allergic mice remained unfed (Table 1, group A). One week after oral tolerization (day 0), all mice were sensitized for a T_H2 response with 5 μ g OVA in 200 μ l of a 1:1 solution of aluminum hydroxide rehydragel (9) (AlOH; Reheis Chemical, Berkeley Heights, NJ, USA): saline intra-peritoneally (i.p.) followed by a 5 μ g dose of OVA in 100 μ l saline i.p. on day 14 (T_H2 priming). Three days after the second i.p. injection (day 17), mice received 1.10^7 KJ1.26 $^+$ $CD4^+$ cells i.v. and were challenged with 70 mg OVA i.g. (secondary T_H2 response) the next day (day 18). A third group of mice, that was only treated for a primary response received 1.10^7 KJ1.26 $^+$ $CD4^+$ cells i.v. 1 day prior to a single dose of 70 mg OVA i.g. (Table 1, group C). To control for the injection of such a large cohort of antigen-specific T-cells, control mice received naive antigen-specific T-cells prior to first antigen encounter (day -8) and were subsequently tolerized (Table 1, group E) or remained unfed (Table 1, group D) prior to priming for a T_H2 response and the induction a secondary T_H2 response with 1 mg of OVA i.g. Blood was collected at indicated time points prior and after i.g. antigenic challenge.

Determination of IgE levels in serum

Both total (ng/ml) and OVA-specific IgE (arbitrary units/ml) serum levels were determined in the serum of T_H2 primed mice during a secondary T_H2 response by ELISA as described elsewhere (9).

Cell division, cytokine producing cells and cytokine secretion

At several time-points after i.g. OVA administration, MLN, PP and spleen were isolated and single cell suspensions were stained for flow cytometry. Cell division was determined based on fluorescence intensity of single CFSE peaks. After 18 h of restimulation with OVA 0.5 mg/ml the percentage of dividing OVA-specific T-cells secreting interleukin (IL)-4, and interferon (IFN)- γ were determined

Table 1. Scheme of the various groups and treatments

Group	Response	Transfer 1.10^7 KJ1.26 $^+$ cells	Tolerization 25 mg OVA i.g.	T_H2 priming 5 μ g OVA i.p. in ALOH in saline		Transfer 1.10^7 KJ1.26 $^+$ cells	Challenge OVA i.g.
A	Secondary			Day 0	Day 14	Day 17	Day 18
B	Secondary (tolerized)		Day - 7	Day 0	Day 14	Day 17	Day 18
C	Primary					Day 17	Day 18
D	Secondary	Day - 8		Day 0	Day 14		Day 18
E	Secondary (tolerized)	Day - 8	Day - 7	Day 0	Day 14		Day 18

using the appropriate murine cytokine secretion assays (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The concentrations of cytokine in culture supernatant of 5×10^6 cells/ml were determined with a BD Cytometric Bead Array according to manufacturers' instructions (BD-Pharmingen).

Capacity of Tr to suppress a secondary immune response

Tr donor mice were tolerized as described above (see also Table 1, group B, E) without receiving KJ1.26⁺ cells. Splenic single cell suspension were prepared at day 25 and enriched for CD4⁺ cells. Acceptor mice that were primed for a primary Th2 response received either 1.10^5 or 1.10^6 Tr in 100 μ l saline i.v. and 1.10^7 naive CD4⁺ KJ1.26⁺ cells and a secondary Th2 response was induced the next day (Table 1, group A). As a control CD4⁺ T cells from saline fed mice were co-transferred with the 1.10^7 naive CD4⁺ KJ1.26⁺ cells. At several time points after challenge, blood was collected for serum IgE levels.

Statistics

The percentage dividing cells was calculated as described by Lambrecht *et al.* (14). Statistically significant differences for serum IgE levels were determined using a MANOVA between individual groups and a Students *t*-test for individual time points. $P < 0.05$ was considered significant.

Results

Naive antigen-specific T-cells enhance IgE levels in a secondary Th2 response

To study the participation of naive T cells in secondary Th2 responses, we transferred naive antigen-specific T cells to three groups of acceptor mice that were Th2 primed with or without prior tolerization and challenged i.g. (Table 1, group A and B respectively), or were only challenged (Table 1, group C).

This established priming regimen with two low dose i.p. antigen administrations and subsequent i.g. challenge results in a highly Th2 skewed response, characteristic of a food-allergic response with prominent IgE levels (9). In addition, to control for the effect on the immune response of infusing a large cohort of antigen-specific T cells a fourth (Th2 control, group D) and fifth (tolerant control, group E) group were designed that received 1.10^7 naive KJ1.26⁺ cells at the start of the experiment, prior to the first antigen encounter. After 8 days in absence of antigen-specific stimulation, these cells could still be detected *in vivo* (Dr D. Krooshoop, personal communication).

A significant increase in OVA-specific (Fig. 1) and total IgE (data not shown) was detected in serum after the induction of a secondary Th2 response but little OVA-specific IgE was detected in serum during a primary response, in tolerant mice, or tolerant control mice (Fig. 1, group B, C, E). Furthermore, the OVA-specific IgE response was significantly higher in Th2 primed mice

Figure 1. Naive antigen-specific T-cells enhance IgE levels in a secondary Th2 response. BALB/c mice were treated as described in Table 1. Briefly, mice were either sensitized (A), tolerized (B) or left untreated (C) prior to transfer of naive KJ1.26⁺ CD4⁺ cells i.v. and challenge with a single i.g. dose of 1 mg OVA in 200 μ l saline. Control groups received naive KJ1.26⁺ CD4⁺ cells at the start of the experiment (day -8) and were subsequently tolerized prior to primary and secondary Th2 response (E) or sensitized for a primary and secondary Th2 response alone (D). The 'no transfer' control mice were immunized according to the same protocol but were not infused with KJ1.26⁺ CD4⁺ cells. OVA-specific serum IgE was determined by ELISA. Arrow indicates time of i.g. challenge. Data are the mean values of at least five mice with SD. *statistically significant ($P < 0.05$).

when the naive antigen-specific T-cells were transferred shortly before the intragastric challenge (group A), compared with the Th2 control mice, which received the KJ1.26⁺ CD4⁺ cells prior to the first antigen encounter (Fig. 1, group D). OVA-specific IgE levels in Th2 control mice (group D) were comparable with those in mice that received no antigen specific T-cells prior to Th2 priming (Fig. 1, 'no transfer').

The results clearly suggest that in a Th2 environment naive T cells can readily be recruited to partake in a secondary immune response and can differentiate into Th2 helper cells as seen by the significant increase in IgE levels, in spite of established memory.

Naive T-cells divide faster during a secondary Th2 response than during a primary Th2 response

To follow the fate of the naive T cells when infused into animals that had undergone different levels of Th2 activation, the cells were labeled with CFSE before

infusion. Acceptor mice were subjected to secondary T_H2 priming with or without prior tolerization (Table 1, group A and B) or to a primary response (Table 1, group C).

The presence and proliferation of OVA-specific T-cells after antigenic challenge was followed in MLN, PP and spleen at 24, 48 and 72 h after i.g. OVA application. In animals undergoing a secondary immune response, vigorous division of the infused naive cells was detected at 48 and 72 h (Fig. 2, group A), in both MLN and PP (data not shown), whereas in the spleen dividing cells were seen from 72 h onwards (data not shown). Already at 48 h the greater majority of the infused cells present in MLN or PP had divided (71% of the $KJ1.26^+ CD4^+$ cells). In animals that had been tolerized before being sensitized for a secondary response (Fig. 2, group B) fewer cells seemed to undergo division (20%), although the total number of divisions was very similar to the nontolerized group (Fig. 2, group A). In primary challenged animals still a

substantial number of divisions could be seen but clearly with different kinetics and numbers of cells involved (only 11% had divided at 48 h after antigen application) (Fig. 2, group C).

These data suggest that naive T-cells divide more rapidly during a secondary T_H2 response, compared with antigen-specific cells in a primary response or tolerized microenvironment as shown by the kinetics of division.

Surface marker expression

Expression of surface markers associated with T cell activation and memory was examined in MLN, PP and spleen after oral antigen administration and was depicted as the relative mean fluorescence intensity (MFI) of surface marker in single peaks of division. At 48 h after the induction of a secondary T_H2 response (Fig. 3, group A) CD25 expression was increased only slightly

Figure 2. Naive T-cells divide faster during a secondary T_H2 response than during a primary T_H2 response. BALB/c mice, tolerized with a single i.g. dose of OVA in (tolerant, Table 1 group B) and control mice (T_H2 , Table 1, group A) were sensitized for a primary T_H2 response by 2 i.p. injections with OVA with a 2 week interval. Three days after the second i.p. injection naive CFSE labeled $KJ1.26^+ CD4^+$ cells were injected i.v. Control naive mice received naive $KJ1.26^+ CD4^+$ cells alone (Table 1, group C). One day after transfer of Tg T cells all mice were challenged for a secondary T_H2 response with OVA i.g. and antigen-specific proliferation was studied in MLN. Percentages indicate $KJ1.26^+ CD4^+$ cells recruited into division. Data are representative of at least five individual mice.

Figure 3. Surface marker expression of dividing antigen-specific T-cells. BALB/c mice were injected and treated as described in Fig. 2, and Table 1. After 48 h MLN cells were stained for KJ1.26 and surface markers. Cell division was determined based on CFSE staining, and mean fluorescence intensity (MFI) of surface marker expression per division is expressed as the relative MFI compared to nondividing antigen-specific T cells in the same sample. The black-bar represents nondividing cells per time-point and each gray-bar represents a separate division: the bar adjacent to the black bar represents the first division and so on. Graphs are the mean of at least three separate experiments with SEM. *statistically significant, ($P < 0.05$) indicates CD62L expression per peak of division is significantly different in T_H2 responders compared with tolerant or primary response groups.

on dividing antigen-specific T-cells, whereas some increase was seen in tolerized animals at later divisions (Fig. 3, group B). In mice undergoing a primary immune response (Fig. 3, group C), a substantial increase of CD25 expression was seen with division. At 72 h, CD25 expression had subsided on most dividing cells in all groups (data not shown).

CD62L expression was readily downregulated upon division with a more gradual decline in the secondary Th₂ response at 48 h after antigen application compared with a primary response or in tolerized mice. The CD62L expression in secondary Th₂ response was significantly different compared to both a primary response or in tolerized mice in all separate peaks of division ($P < 0.05$), while no significant differences were observed between a primary response and in tolerized mice. Even though CD69 and CD45RB followed a pattern as seen for other T cell responses, no differences in kinetics of expression between any of the three groups were found (data not shown).

In sum, during a secondary Th₂ response naive T cells differentiated into T cells with an activated/memory like phenotype with a relatively low expression of CD25 and downregulated expression of CD62L.

Naive antigen-specific T-cells contribute to enhanced cytokine release during a secondary Th₂ response

To assess the functional differentiation of the naive antigen-specific T cells during a secondary Th₂ response, cytokine profiles were measured. Already at 24 h after the induction of a secondary Th₂ response, MLN cells produced large amounts of IL-2 (Fig. 4A, group A) compared with the MLN cells of tolerized mice and mice undergoing a primary immune response (Fig. 4A, group C), which correlated with the more rapid onset of T-cell division at this site in mice undergoing a secondary Th₂ response. At later time points no differences in IL-2 production could be found. In the spleen of both secondary and secondary tolerized mice, but not in the case of primary challenged mice, substantial IL-2 production was seen already at early time points (data not shown), possibly correlating with the preferred presence of memory cells in this organ. Cytokines that have been related to a specific Th₂ response, such as IL-4 and IL-5 were only detectably secreted by MLN and spleen cells isolated during a secondary Th₂ response (Fig. 4A, group A). IFN- γ could be detected in the supernatant of MLN and spleen cell cultures of mice during a secondary Th₂ response, but seemingly at lower concentrations than in the tolerant mice or in a primary immune response. To identify whether the expanding transferred cells were a source of these cytokines and to assess their polarity under different conditions of priming, the percentage of dividing cytokine secreting cells was determined. As seen in Fig. 4B, OVA specific T cells that divided during a secondary Th₂ response (group A) were predominantly

Figure 4. Naive antigen-specific T-cells contribute to an enhanced cytokine release during a secondary Th₂ response. BALB/c mice were injected and treated as in Fig. 2, and Table 1. At 24, 48 and 72 h after the secondary Th₂ challenge MLN-cells were re-stimulated *in vitro* for 72 h with OVA. (A) Cytokines in supernatant are represented as mean concentration in pg/ml of at least three separate experiments, with SEM. (B) IL-4 and IFN- γ producing cells were determined at 48 h after the secondary Th₂ challenge. For analysis, the percentage of secreting cells within the dividing KJ1.26 CFSE⁺ cell population was determined after an overnight restimulation with OVA. Data are represented as mean percentage of cytokine-secreting antigen-specific T cells per peak of division of at least three separate experiments, with SEM. The black bar represents nondividing cells and the grey bars the subsequent dividing numbers from left to right.

IL-4 producing suggesting they had differentiated towards a Th₂ phenotype.

In sum, MLN cells in Th₂ primed mice had a Th₂ related cytokine profile based on IL-4 and IL-5 secretion, and produced IL-2 at earlier time points compared to MLN cells of naive and tolerized mice.

Tr can suppress a secondary Th₂ response *in vivo*

Mucosal Tr can suppress a primary Th₂ response upon adoptive transfer. These Tr are present in the CD4⁺ spleen T-cell population at 7 days after the intragastric antigenic challenge of animals that have undergone the tolerizing protocol as in group B (but without the infusion of KJ1.26 cells). However, it is unclear whether these mucosal Tr are capable of suppressing a secondary Th₂ response, when both memory and naive T cells are

Figure 5. Mucosal Tr can suppress a secondary Th2 response. Tr (squares), and naive T-cells (black circles) were isolated from spleen of tolerant (comparable with Table 1, group E, only without KJ1.26⁺ cells) and naive donor mice respectively. Single cell suspensions were enriched for CD4⁺ T cells. Either 1.10^5 (grey squares), 1.10^6 (white squares) Tr or 1.10^6 naive T-cells (black circles) were transferred to Th2 primed recipient mice by i.v. injection and 1 day later the mice were challenged i.g. for secondary Th2 response. Arrow indicates time of challenge. Data are represented as the relative increase in OVA-specific IgE compared with 1 day prior to challenge of five separate mice, with SEM. *statistically significant ($P < 0.05$).

present and can actively contribute to the immune response. Therefore, mucosally induced CD4⁺ Tr were adoptively transferred together with 1.10^7 naive antigen-specific T cells to Th2 primed acceptor mice (group A) directly prior to a secondary Th2 response (day 17).

As shown in Fig. 5, the increase in OVA-specific IgE could dose dependently be suppressed, as shown by the decreased IgE response in mice receiving 1.10^6 CD4⁺ mucosal Tr prior to a secondary Th2 response, but not after transfer of 1.10^5 CD4⁺ Tr.

In conclusion, mucosal Tr can indeed suppress a secondary Th2 response upon adoptive transfer even in the presence of a large cohort of naive antigen-specific T cells. Although statistical analysis is difficult due to the high variation in the IgE response of mice receiving naive T-cells alone, this result implies that the difficulty to induce oral tolerance in established disease is not because of the inability of mucosal Tr to suppress memory Th2 cell function upon antigenic challenge.

Discussion

Here we show that naive CD4⁺ T-cells actively contribute to a secondary Th2 response. Upon antigen encounter, these cells are rapidly activated, divide and enhance the humoral IgE response despite the presence of memory T cells, recognizing the same antigen. The active expansion of these naive T cells is larger than that of cells in a

primary response despite a relatively lower expression of IL-2 receptor- α (CD25). This proliferation may be instigated by the presence of large quantities of IL-2 seen in MLN, as it was recently demonstrated that exogenous IL-2 enhances both the number of cells entering division as well as the kinetics of division (15). Indeed, the IL-2 present in the MLN of mice with a secondary infection may at least partly originate from memory T cells, since a large proportion of non-Tg CD45⁺ KJ1.26⁻ cells produced IL-2 in secondary Th2 responders (data not shown). The finding that developing naive T-cells largely differentiate into IL-4 producing cells and ultimately enhance the IgE response suggests that they can differentiate into a Th2 like phenotype to become helper cells for Ig production. This differentiation is likely to be skewed by the presence of local IL-4 that may directly affect the naive T-cell or act indirectly by modulating DC activity (16).

Despite a highly Th2 skewed microenvironment the enhancement of the secondary IgE response induced by the infusion of large cohorts of naive T cells can be halted when sufficient numbers of mucosal Tr are present (Fig. 5). The mechanism of the suppression is unclear, but one could speculate that suppression of both primary and secondary responses requires a similar mechanism. Previously, we have shown that upon adoptive transfer mucosal Tr convert naive T cells of the acceptor mouse into Tr cells, a phenomenon described as 'infectious tolerance' (17). As this form of propagation of tolerance requires the involvement of naive T cells such a mechanism of suppression would shed new light on the effectiveness of mucosal tolerance in suppression of established disease. Primarily, it requires the presence of sufficient naive T cells, which may be a limiting factor in case tolerance is generated in an already actively sensitized environment, where existing memory has to be overruled. This is related to differences in homeostatic potential of naive and memory cells, as seen in the reduced sensitivity of memory cells for activation induced cell death and the decreased dependency on antigenic stimulation for survival (18). Both are intrinsic factors for the immune system to react avidly to secondary challenges, but clearly underlie the problems encountered in therapeutic applications of tolerance induction. Indeed, perturbation of the naive T-cell repertoire because of distorted or reduced thymic export has been associated with autoimmune disease where therapeutic application of oral tolerance has not been successful such as multiple sclerosis and rheumatoid arthritis (19, 20). Furthermore, also reduced susceptibility to oral tolerance in a primary response in older mice could be related to a defective naive T-cell pool resulting from thymus atrophy (21). The factors with which mucosal Tr suppress T-cell responses have not been fully elucidated and are under current investigation in our laboratory. Suppression may be achieved by secretion of anti-inflammatory cytokines such as IL-10 and TGF- β , which may influence either the antigen-presenting cell, the

memory T_H2 cell or the naive T-cells directly (22–26). In the experiments described in this study, we have used T cells with a transgenic T-cell receptor recognizing one dominant peptide of OVA. It should be taken into account that during a secondary allergic reaction the responding naive T cells may recognize a different epitope than that recognized during first antigen encounter, generally denoted as epitope spreading. Such a phenomenon may further complicate tolerance induction during a secondary allergic response.

In sum, we report that naive $CD4^+$ T cells contribute to a secondary T_H2 response and that these cells will

differentiate accordingly. Moreover, mucosal Tr cells are capable of suppressing such an established immune response. These data will be of importance for possible applications of Tr and tolerance induction in immunotherapy.

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