

# Immunization with mannosylated peptide induces poor T cell effector functions despite enhanced antigen presentation

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## Abstract

**In this study, we investigated the development of T cell responses in mice after administration of a mannosylated ovalbumin peptide (M-OVA<sub>323–339</sub>). Immunization with M-OVA<sub>323–339</sub> in complete adjuvant resulted in enhanced antigen presentation in draining lymph nodes. Monitoring the fate of CFSE-labeled ovalbumin peptide-specific TCR transgenic CD4<sup>+</sup> T cells revealed that immunization with M-OVA<sub>323–339</sub> induced normal clonal expansion, recirculation and CD62L expression of antigen-specific T cells *in vivo*. However, these T cells developed only poor effector functions, reflected by minimal IFN- $\gamma$  production, low IgG2a levels in serum and poor peptide-specific delayed-type hypersensitivity (DTH) responses. This diminished inflammatory response was associated with decreased infiltration of T cell blasts and macrophages. Importantly, also mice with functional effector T cells did not mount a robust DTH response after a challenge with M-OVA<sub>323–339</sub> in the ear, although their T cells responded normally to M-OVA<sub>323–339</sub> *in vitro*. In conclusion, mannosylated peptide induces proliferation of T cells with impaired T<sub>H</sub>1 cell effector functions and additionally abrogates the activity of pre-existing effector T cells.**

## Introduction

Antigen-presenting cells (APCs) are well equipped to discriminate between self and non-self via the expression of pathogen recognition receptors. The balance between stimulatory and inhibitory signals evoked by triggering these receptors enable APC to fine-tune immune responses (1). Toll-like receptors can recognize a wide range of conserved molecular motifs of pathogens, which can result in APC maturation and the induction of a powerful immune response (2). Recognition of glycosylated self and non-self antigens is mediated by C-type lectin receptors (CLRs) (3). These receptors are highly expressed on immature APC and several studies indicate that CLR targeting may result in immune suppression and tolerance instead of immunity.

Studies by Apostolopoulos *et al.* (4, 5) have revealed that a tumor antigen conjugated to mannan is efficiently internalized by the mannose receptor, expressed on murine dendritic cells (DCs) and macrophages, resulting in MHC class I- or MHC class II-restricted presentation, depending on the applied form of mannan. In line with this, we have previously

shown *in vitro* that endocytosis of bis-mannosylated peptides by human DC is mediated via the mannose receptor, resulting in very efficient uptake and presentation in MHC II (6). However, applying this strategy of receptor-mediated uptake *in vivo* did not result in the development of full-blown immunity. Instead, immunization with mannosylated self-peptide induced antigen-specific tolerance to experimental autoimmune encephalomyelitis (EAE) in mice. The presence of complete adjuvant containing *Mycobacterium tuberculosis* could not break this tolerance, indicating that this might be a very powerful strategy to silence autoimmunity. Immunization with mannosylated self-peptide resulted in poor T cell effector functions, resulting in reduced delayed-type hypersensitivity (DTH) responses and the absence of EAE symptoms. Paradoxically, antigen-specific T cells were present *in vivo* and were responsive to antigen-specific stimulation *in vitro* (7). Several others have shown that members of the CLR family can modulate the immune response. For example, *in vivo* targeting of immature mouse

DC can be achieved by antigen delivery via DEC-205. Fusion proteins consisting of an anti-DEC-205 antibody and a T cell epitope of ovalbumin induced immunological unresponsiveness both to class I- and class II-restricted T cell epitopes (8, 9). Similarly, targeting of myelin antigens toward DEC-205 was used to induce peripheral tolerance in EAE (10). It has also been shown that various pathogens can target DC-SIGN, expressed on DC, to circumvent intracellular degradation and antigen presentation, and prevent their elimination by the host (11–14).

Here, we studied the immune reaction in response to a mannosylated ovalbumin peptide (M-OVA), employing TCR transgenic CD4<sup>+</sup> T cells specific for ovalbumin peptide (OVA<sub>323-339</sub>) (15, 16). Immunization with M-OVA<sub>323-339</sub> induced enhanced antigen presentation in draining lymph nodes (LNs) and resulted in apparently normal expansion and recirculation of OVA<sub>323-339</sub>-specific T cells. However, the effector functions of T cells were impaired, evident from poor DTH responses in conjunction with little infiltration of T cell blasts and macrophages at the site of antigen challenge.

## Methods

### Animals

Female C57BL/6 mice and B6.SJL mice were purchased from Charles River (Maastricht, Netherlands). OTII and DO11.10 mice on a C57BL/6 background were bred in our own facility. Animals were housed under standard conditions with free access to food and water. All experimental procedures were approved by the Animal Welfare Committee.

### Peptide synthesis

OVA<sub>323-339</sub> (ISQAVHAAHAEINEAGR) and its mannosylated form (M-OVA<sub>323-339</sub>) were synthesized as described elsewhere (6, 17). In short, the peptides were prepared using solid-phase synthesis. Mannosylation was accomplished by N-terminal elongation of the peptide with a building block containing lysine coupled to two tetraacetyl-protected mannose groups. The OVA<sub>323-339</sub> peptide was elongated with bis-acetyl lysine only. The acetyl protecting groups on the mannose moieties were removed using Tesser's base. All peptides were analyzed with Maldi-Tof mass spectrometry and showed the expected masses.

### In vitro cultures

Spleens were isolated from OTII or DO11.10 mice and single-cell suspensions were prepared through a 40- $\mu$ m filter (BD Falcon, Bedford, MA, USA). For CFSE labeling, the cells were incubated with 5  $\mu$ M CFSE in RPMI 1640 (Cambrex, East Rutherford, NJ, USA) for 10 min and subsequently washed twice with ice-cold PBS, as has been published by Lyons *et al.* (18). To study *in vitro* proliferation, cells were resuspended in RPMI 1640 containing 5% FCS (Cambrex), 100 U ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin, 10 mM ultraglutamine and 50  $\mu$ M  $\beta$ -mercaptoethanol and seeded in 96-well flat-bottom plates (Costar, Cambridge, MA, USA) at a density of  $2 \times 10^5$  cells per well. The OTII cells were stimulated with 0.1–100  $\mu$ g ml<sup>-1</sup> peptide for 4 days. For long-term cultures, OTII and DO11.10 cells were stimulated with

3–30  $\mu$ g ml<sup>-1</sup> peptide for 3 days and subsequently expanded in the presence of 15 U ml<sup>-1</sup> IL-2 for 7 days. The recall response of LN cells isolated from immunized C57BL/6 mice was determined after 3 days of stimulation with 30  $\mu$ g ml<sup>-1</sup> OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>.

For localization of *in vivo* antigen presentation, C57BL/6 mice were euthanized and single-cell suspensions were prepared from spleen and LNs. To isolate cells from ears, skin layers were separated and incubated for 1 h at 37°C with collagenase type IV (Worthington, Lakewood, NJ, USA) before preparing a single-cell suspension. Either of these cells were irradiated (30 Gy) and used as APC presenting endogenous antigen to naive CD4<sup>+</sup> OTII cells for 3 days at a ratio of 2 to 1, without further addition of peptide. Naive CD4<sup>+</sup> OTII cells were isolated from the spleen via negative selection with Dynal beads (Dynal Biotech, Oslo, Norway), resulting in a 80–85% pure CD4<sup>+</sup> T cell population. Supernatants were collected from the cultures and proliferation was assessed by addition of 0.5  $\mu$ Ci per well of [<sup>3</sup>H]thymidine (2Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) for 6 h.

### In vivo transfer of OTII cells

OTII mice were sacrificed and spleens and LNs were isolated. A single-cell suspension was prepared in RPMI medium and incubated on a nylon wool column for 45 min. This procedure depleted 50% of B cells and macrophages and resulted in an enriched cell suspension containing 50% CD4<sup>+</sup> T cells. Cells were washed extensively with PBS before transfer into C57BL/6 or B6.SJL mice. In some experiments, OTII cells were labeled with CFSE before transfer as described above. Intravenous injection of  $5 \times 10^6$  CFSE-labeled OTII cells into mice was performed 1 day before immunization.

### Immunization protocol

Mice (7–10 weeks old) were immunized subcutaneously with 100  $\mu$ g OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> dissolved in PBS and emulsified in an equal volume of complete adjuvant supplemented with 1 mg ml<sup>-1</sup> *M. tuberculosis* (H37RA; Difco Laboratories, Detroit, MI, USA). Control mice were immunized with PBS in adjuvant only.

### Delayed-type hypersensitivity

The DTH response was evaluated by injecting 12 nmol of OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>, dissolved in 10  $\mu$ l saline, into the dorsal side of the right ear of mice using a Hamilton syringe fitted with a 30-gauge needle. As a control for non-specific ear swelling, 10  $\mu$ l of saline was injected into the left ear. Ear thickness was measured before and 24 h after intradermal injection using a Mitutoyo micrometer. Results are expressed as the percentage-specific ear swelling, obtained by subtracting the percentage non-specific ear swelling.

### Flow cytometry

For FACS analysis, cells were collected from *in vitro* cultures, or single-cell suspensions were prepared from isolated organs. Blood samples were treated with lysing solution (Pharmingen, San Diego, CA, USA) before staining. To isolate

cells from ears, these were incubated with collagenase type IV (Worthington) for 1 h before homogenization. Lungs and livers were isolated after perfusion of mice with PBS and incubated with collagenase type IV as described above. Subsequently, a Ficoll gradient was used to isolate cells from these organ homogenates. Cells were stained with different rat-anti-mouse antibodies obtained from Pharmingen (CD3-FITC, CD4-PE, CD4-PerCP, CD25-biotin, CD45.2-PerCP, CD62L-Allophycocyanin, IFN- $\gamma$ -Alexa647, IL-10-FITC, IL-4-Alexa647, IL-5-PE and F4/80-Allophycocyanin). Where needed, isotype-matched controls were included (rat-anti-mouse IgM-biotin, rat-anti-mouse IgG1-PE and -Allophycocyanin, rat-anti-mouse IgG2a-Allophycocyanin and rat-anti-mouse IgG2b-Alexa647). Biotinylated antibodies were visualized with Streptavidin-APC. For intracellular FACS staining, cells were incubated at 4–6 h with  $10 \mu\text{g ml}^{-1}$  OVA<sub>323-339</sub> or phorbol myristate acetate/ionomycin in the presence of Brefeldin A (Sigma, Zwijndrecht, The Netherlands). Fixation and permeabilization reagents (Caltag Laboratories, Burlingame, CA, USA) were used for intracellular staining.

#### The ELISA

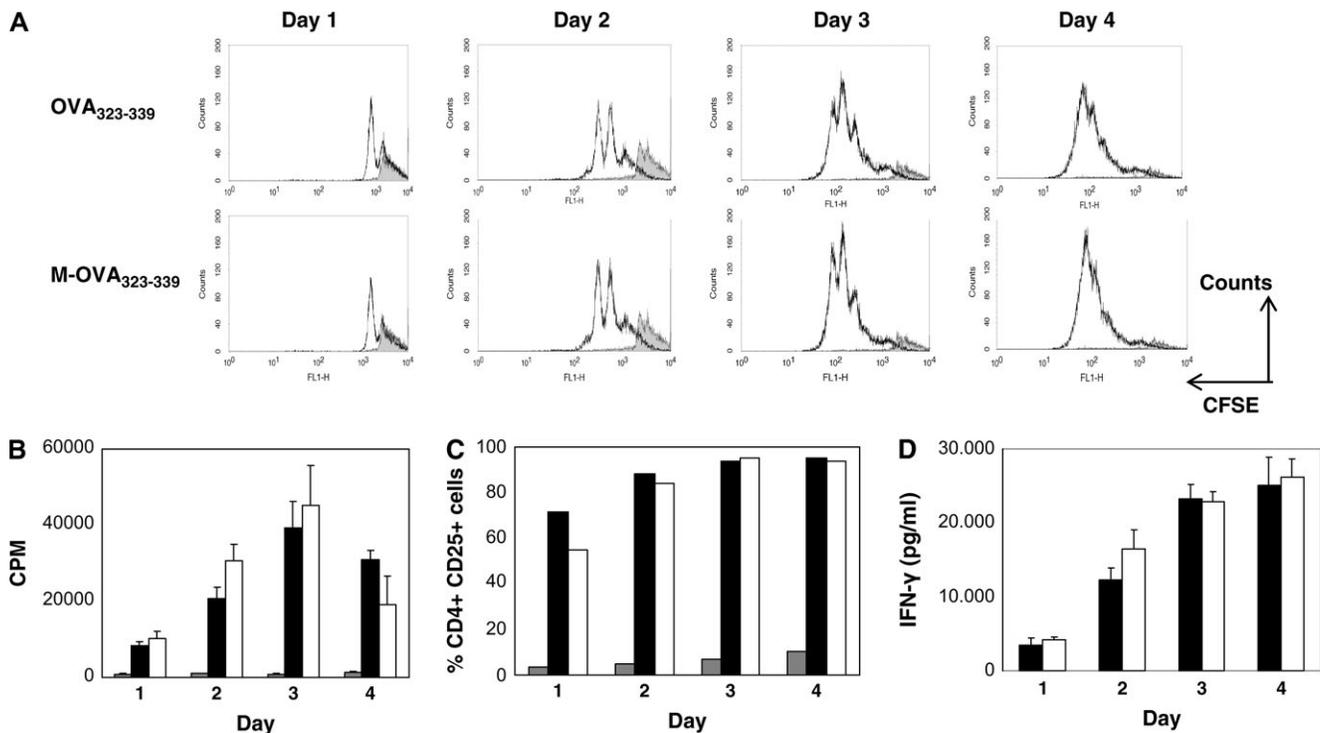
IFN- $\gamma$ , IL-4 and IL-10 production was measured in supernatants by ELISA. For IFN- $\gamma$  antibody, R46A2 (American Type Culture Collection [ATCC] HB170) was used as capture antibody and biotinylated AN18 (kindly provided by Anne O'Garra, DNAX) as detection antibody. For IL-4 antibody,

11B1 (ATCC HB188) was used as capture antibody and biotinylated BVD4 (kindly provided by Jon Abrams, DNAX) as detection antibody. For IL-10 antibody, 2A5 was used as capture antibody and biotinylated SXC-1 as detection antibody (both obtained from Pharmingen). An ELISA plates with high binding capacities (Nunc, Roskilde, Denmark) were coated overnight with antibodies properly diluted in carbonate buffer, pH 9.5, or phosphate buffer, pH 6.5. After blocking with PBS containing 0.05% Tween and 0.02% gelatin (PTG), plates were incubated with supernatant samples (diluted in culture medium). After washing, detection antibodies diluted in PTG were added to the plates. Subsequently, plates were incubated with Streptavidin-polyHRP (Sanquin, Amsterdam, The Netherlands) and developed using 3,3',5,5'-tetramethylbenzidine (Sigma). The color reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and plates were read at 450 nm using a Versamax microplate reader. Standard curves were prepared using recombinant cytokines.

The detection of OVA-specific IgG antibodies in serum was performed as described elsewhere (19) and for in this study plates with high binding capacities were coated overnight with  $2 \mu\text{g ml}^{-1}$  OVA<sub>323-339</sub> dissolved in PBS.

#### Statistics

Statistical analysis of data was performed with the Mann-Whitney *U*-test.



**Fig. 1.** Mannosylated peptide induces normal *in vitro* proliferation of OTII splenocytes. OTII splenocytes were cultured for 4 days in the presence of  $10 \mu\text{g ml}^{-1}$  OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> or with medium alone. Cell division of CD4<sup>+</sup> T cells was evaluated via dilution of CFSE signal (A; the gray histograms represent the medium control) and incorporation of [<sup>3</sup>H]thymidine (B; filled squares, OVA<sub>323-339</sub>; open squares, M-OVA<sub>323-339</sub>; medium control in gray). CD25 expression by CD4<sup>+</sup> OTII cells was evaluated and numbers of positive cells are indicated (C; filled bars, OVA<sub>323-339</sub>; open bars, M-OVA<sub>323-339</sub>; medium control in gray). IFN- $\gamma$  production levels in supernatants collected on days 1–4 was evaluated in duplicate by ELISA (D). Similar results were obtained at the other peptide concentrations tested. Representative data from four different experiments are shown.

## Results

### Mannosylated peptide is well recognized by OTII splenocytes *in vitro*

To study whether peptide mannosylation influenced antigen recognition by OTII cells *in vitro*, we cultured CFSE-labeled OTII splenocytes for 4 days with different concentrations (0.1–100  $\mu\text{g ml}^{-1}$ ) of OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>. Cell division of gated CD4<sup>+</sup> T cells was monitored daily by dilution of CFSE signal (Fig. 1A) and by [<sup>3</sup>H]thymidine incorporation (Fig. 1B). In general, OTII splenocytes proliferated equally to OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> at all peptide concentrations tested. Results after stimulation with 10  $\mu\text{g ml}^{-1}$  peptide are depicted. Maximal proliferation was observed after 3 days of culture and no peptide-related differences were found. As depicted, medium-stimulated OTII cells showed no detectable proliferation.

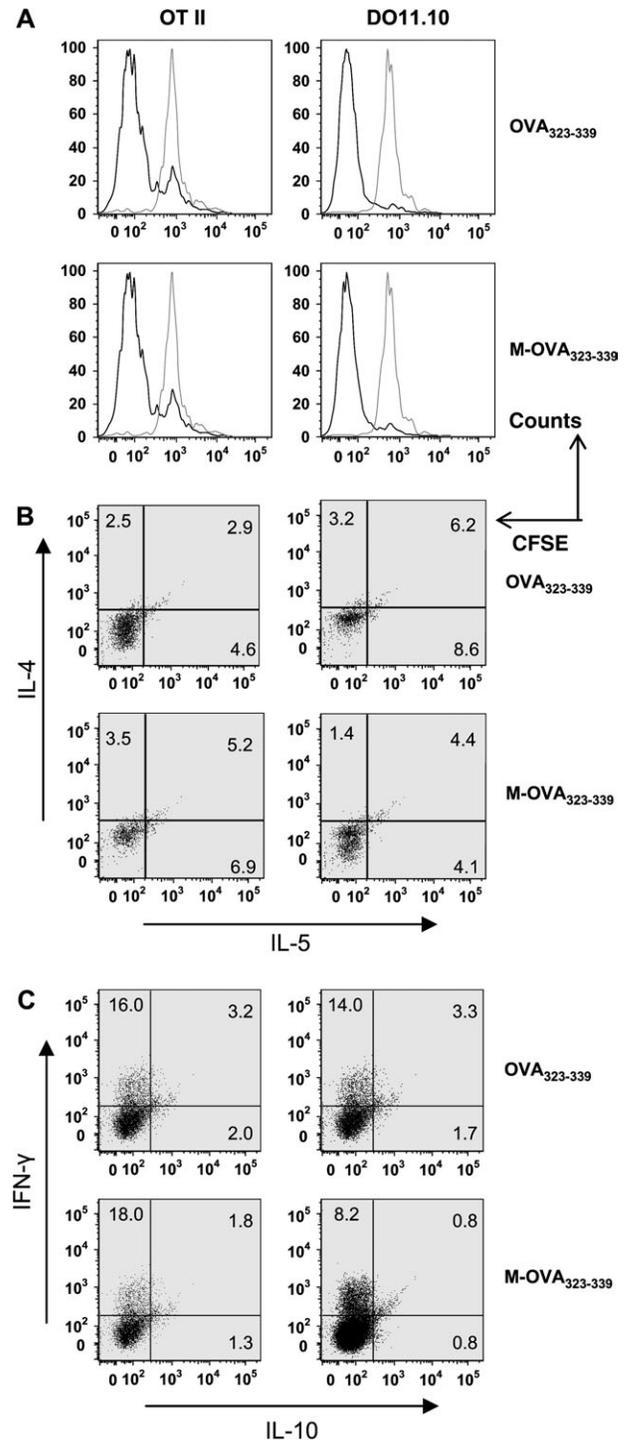
We studied the activation state of CD4<sup>+</sup> OTII cells via their CD25 expression and as presented in Fig. 1(C), similar expression of CD25 was detected after stimulation with either peptide, reaching up to 90% positive cells on day 4. ELISA on culture supernatants revealed that both *in vitro* stimulation with OVA<sub>323-339</sub> and M-OVA<sub>323-339</sub> induced considerable IFN- $\gamma$  production (Fig. 1D). The levels of IL-4 were below the detection limit (<8 pg ml<sup>-1</sup>) under all conditions; stimulation with the highest concentration of OVA<sub>323-339</sub> induced some IL-10 production, while the mannosylated peptide did not (data not shown). Staining for annexin-V revealed that stimulation with either peptide did not induce apoptosis of OTII cells (data not shown).

To address the possible development of T<sub>H</sub>2 cells, which requires T cell progression through multiple cell divisions for full differentiation (20), we cultured CFSE-labeled OTII and DO11.10 splenocytes for a prolonged period of time. After 3 days of antigen-specific stimulation, we observed that DO11.10 cells had responded more vigorously to both peptides, as compared with OTII cells (Fig. 2A). We further expanded the cells with IL-2 for one more week and on day 7 (data not shown) and day 10, cytokine production was determined by intracellular FACS staining. Both OTII and DO11.10 cells developed substantial numbers of IFN- $\gamma$ -producing cells regardless of stimulation with mannosylated or non-mannosylated peptide; IL-4, IL-5 and IL-10 production was undetectable (Fig. 2B and C).

Altogether, we conclude that *in vitro* stimulation with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> induced equal proliferation of OVA<sub>323-339</sub>-specific CD4<sup>+</sup> T cells and resulted in the development of a T<sub>H</sub>1 phenotype.

### Immunization with mannosylated peptide results in an impaired T<sub>H</sub>1 response

To evaluate whether immunization with mannosylated peptide induced qualitatively different T cell responses *in vivo*, the frequency of naive OVA<sub>323-339</sub>-specific T cells was first increased in C57BL/6 mice by injection of enriched OTII cells. One day later, these mice were immunized with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> in CFA. Five days after immunization, the ability of the mice to mount a DTH response to soluble OVA<sub>323-339</sub> was determined. We observed



**Fig. 2.** Stimulation with mannosylated peptide does not support T<sub>H</sub>2 differentiation. CFSE-labeled DO11.10 and OTII splenocytes were cultured for 3 days in the presence of 10  $\mu\text{g ml}^{-1}$  OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> or medium alone. Cell division of CD4<sup>+</sup> T cells was evaluated via dilution of CFSE signal on day 3 (A; the gray histogram represents the medium control). Subsequently, the cells were expanded in the presence of IL-2 for another week and on day 10, the production of cytokines was determined by intracellular FACS staining. DO11.10 cells as well as OTII cells showed no IL-4 and IL-5 staining (B), while considerable numbers of IFN- $\gamma$ -producing cells were observed in the absence of IL-10 production (C). Similar results were obtained at other peptide concentrations tested.

a tendency toward decreased ear swelling in M-OVA<sub>323-339</sub>-immunized mice as compared with OVA<sub>323-339</sub>-immunized mice ( $P = 0.12$ ). A significant difference was observed when the mice were challenged again on day 26 (Fig. 3A,  $P < 0.01$ ).

After sacrifice on day 35, the LNs of the mice were collected and the *in vitro* recall response to both peptides was determined. Despite the reduced DTH reactivity *in vivo*, equal proliferation was observed in mice immunized with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>, independent from the peptide used for stimulation (Fig. 3B).

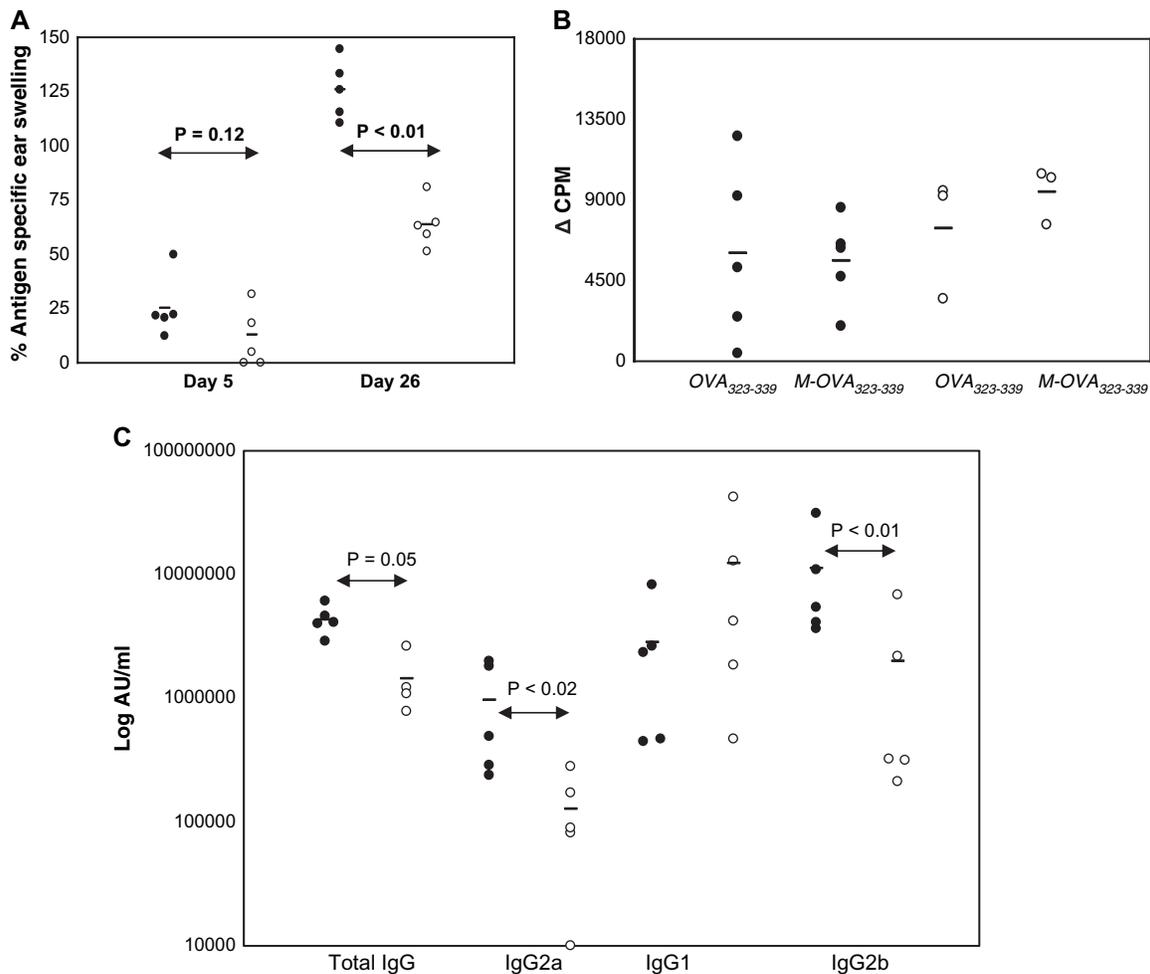
Analysis of the levels of OVA<sub>323-339</sub>-specific IgG antibodies in the sera of these mice revealed that significantly less peptide-specific IgG was detectable in M-OVA<sub>323-339</sub>-immunized mice ( $P < 0.05$ ) due to lower levels of IgG2a and IgG2b (Fig. 3C).

Also in mice that were sacrificed on day 16 after immunization with M-OVA<sub>323-339</sub>, equal *in vitro* responses of LN cells were observed and significantly lower levels of

OVA<sub>323-339</sub>-specific IgG2a, although the overall response at this earlier time point was less vigorous (data not shown). These data are supportive for decreased T<sub>H</sub>1 reactivity in the absence of an increased T<sub>H</sub>2 response.

#### Mannosylated peptide induces enhanced antigen presentation *in vivo*

Antigen presentation after immunization with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> in adjuvant containing *M. tuberculosis* was next evaluated in C57BL/6 mice. For this purpose, LNs draining the immunization site, (non-draining) mesenteric LN and spleens were isolated 1–10 days after immunization of these mice. The isolated cells were irradiated and used as APC presenting endogenous peptide to naive CD4<sup>+</sup> OTII cells without further addition of antigen. CD4<sup>+</sup> OTII cells showed minimal proliferation (<1000 counts per minute) when co-cultured with irradiated cells from sham-immunized mice (data not shown). Particularly, cells from the draining



**Fig. 3.** Immunization with mannosylated peptide induces poor T<sub>H</sub>1 responses. C57BL/6 mice were immunized with OVA<sub>323-339</sub> (filled circles) or M-OVA<sub>323-339</sub> (open circles) and DTH responses to OVA<sub>323-339</sub> were evaluated on day 5 and day 26 (A). Immunization with M-OVA<sub>323-339</sub> resulted in a significantly reduced DTH response on day 26 ( $P < 0.01$ ). Mice were sacrificed on day 35 and draining LN cells were cultured *in vitro* with  $10 \mu\text{g ml}^{-1}$  OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> (B). All animals showed similar proliferation, regardless of the peptide used for stimulation. OVA<sub>323-339</sub>-specific IgG antibodies levels in serum were determined by ELISA (C). Immunization with M-OVA<sub>323-339</sub> resulted in significantly lower IgG titers ( $P < 0.05$ ) because IgG2a levels ( $P < 0.02$ ) and IgG2b levels ( $P < 0.01$ ) were reduced. Group means are indicated.

LN induced proliferation of naive OTII cells, regardless of the peptide used for immunization, indicating that this was a major site of antigen presentation *in vivo*. Immunization with M-OVA<sub>323-339</sub> induced enhanced antigen presentation on days 1 and 3 (Fig. 4A).

In a similar way, antigen presentation after local administration of soluble mannosylated peptide was evaluated. For this purpose, soluble OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> or PBS was injected into the ears of C57BL/6 mice and 1 day later, the draining (cervical) LN, (non-draining) inguinal LN and the ears were used as a source of APC. After irradiation, these cells were co-cultured with naive CD4<sup>+</sup> OTII cells without further addition of peptide. Injection of soluble M-OVA<sub>323-339</sub> into the ear induced increased antigen presentation in the draining LN 24 h after administration (Fig. 4B,  $P < 0.05$ ), as opposed to injection of OVA<sub>323-339</sub>. Part of the mannosylated antigen remained localized in the ear and was presented there by local APC to the same extent as OVA<sub>323-339</sub> (Fig. 4C,  $P < 0.05$  and  $P = 0.08$ , respectively, as compared with PBS controls).

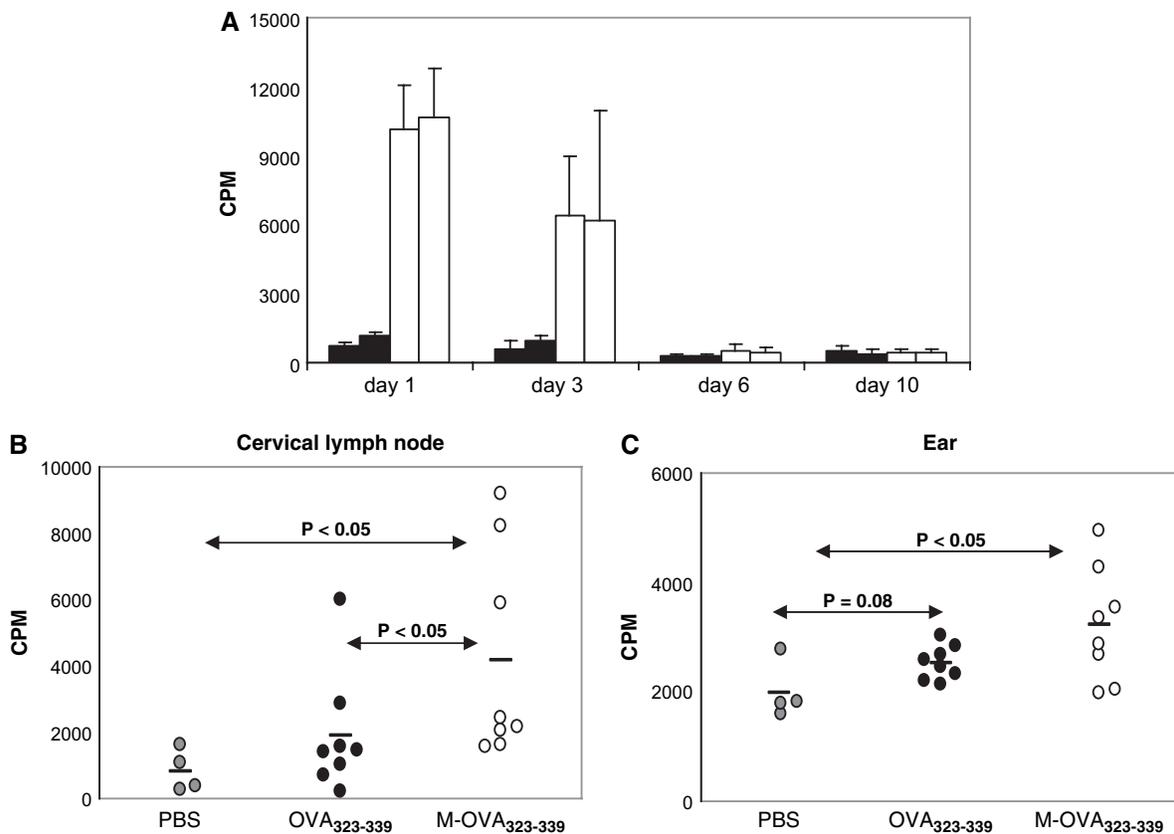
In both experiments described above, substantial presentation of mannosylated peptide, but not the normal peptide, was observed in non-draining LN (data not shown).

In conclusion, both peptides were equally presented in tissue, while presentation of the mannosylated peptide in the (draining) LN was enhanced for a prolonged period of time.

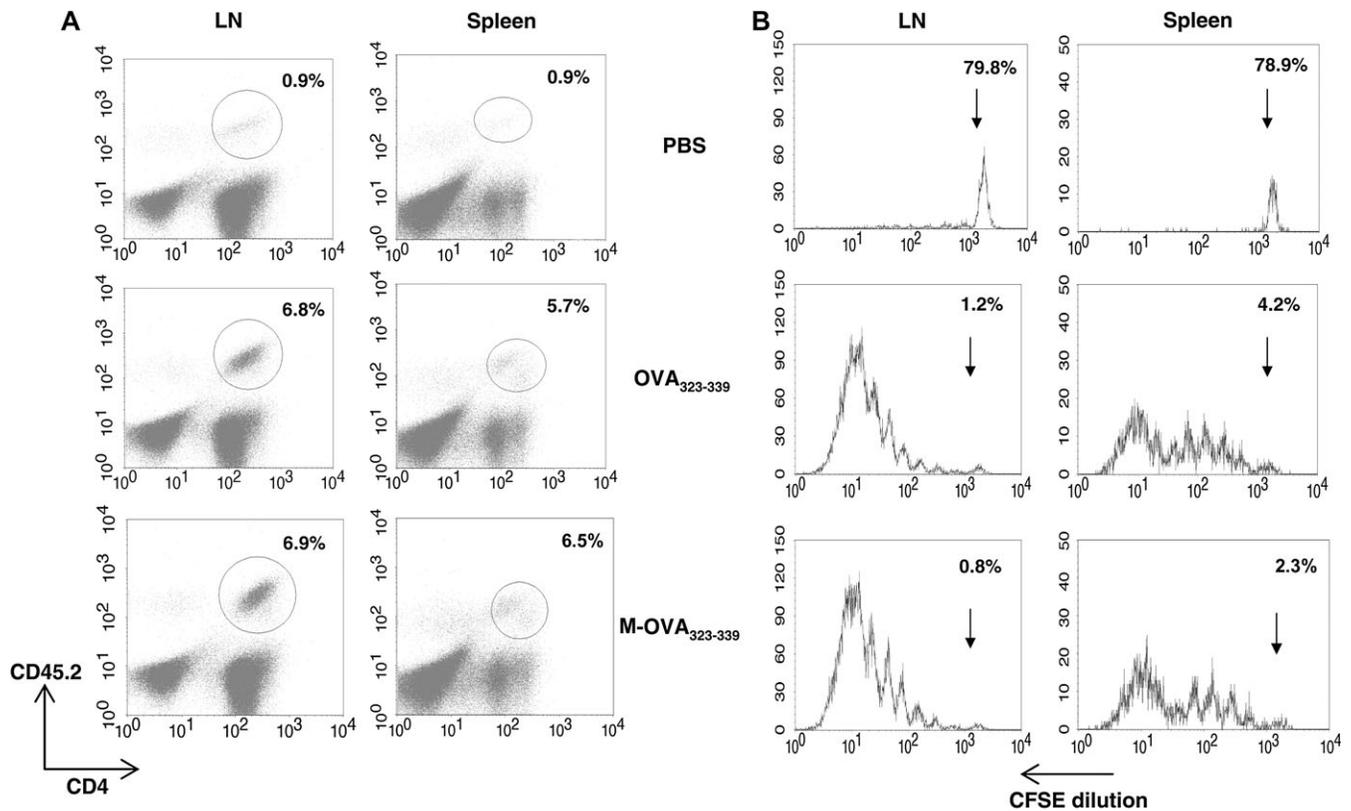
#### Similar expansion and circulation of OTII cells *in vivo* after immunization with mannosylated peptide

To evaluate the clonal expansion of T cells in response to immunization with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>, CFSE-labeled OTII cells were transferred into B6.SJL mice (CD45.1<sup>+</sup>), which enabled retrieval of OTII cells based on expression of the congenic marker CD45.2. Control mice were immunized with PBS in complete adjuvant.

Draining LN and spleens were isolated on days 4, 5 and 6 after immunization. Representative data obtained on day 4 are presented in Fig. 5. Immunization with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> resulted in the recruitment of similar numbers of OTII cells both to draining LN and spleens (Fig. 5A). The



**Fig. 4.** Mannosylated peptide induces enhanced antigen presentation *in vivo*. C57BL/6 mice were immunized with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> and on each indicated time point, two mice were sacrificed (represented by individual bars). To detect antigen presentation, the draining LN were isolated, irradiated and co-cultured with naive CD4<sup>+</sup> OTII cells that were isolated via negative selection with Dynal beads and were 80–85% pure. No further antigen was added (A; filled bars, OVA<sub>323-339</sub>; open bars, M-OVA<sub>323-339</sub>). Mean data from quadruplicates are presented as counts per minute (c.p.m.)  $\pm$  SD. One out of three similar experiments is presented. (B and C) They show the responses of C57BL/6 mice that received an intra-dermal injection with PBS, OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> into the ear. One day later, cell suspensions were prepared from the cervical LN and ears. The cells were irradiated and co-cultured with naive CD4<sup>+</sup> OTII cells to detect antigen presentation. Proliferation was assessed in triplicates and evaluated by [<sup>3</sup>H]thymidine incorporation (c.p.m.  $\pm$  SD). Results from two combined experiments are presented and group means are indicated.



**Fig. 5.** Normal *in vivo* expansion of OTII cells after immunization with M-OVA<sub>323-339</sub>. CFSE-labeled OTII cells were transferred into B6.SJL mice and these were immunized 1 day later with PBS, OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>. Mice were sacrificed on day 4 after immunization and draining LN and spleens were isolated. The percentage of CD45.2<sup>+</sup> OTII cells within the population of CD4<sup>+</sup> T cells was determined by flow cytometry. These cells were gated and percentages are presented (A). Cell division was visualized via dilution of the CFSE signal. The percentages of undivided cells are indicated (B). Similar results were obtained on days 5 and 6. Three mice per group were studied and representative data are shown.

division pattern of these OTII cells is depicted in Fig. 5(B) and we observed that highly divided OTII cells were particularly situated in the draining LN, while these cells in the spleen were less proliferative. No peptide-related differences were observed. Upon sham immunization, only low numbers of OTII cells (<1%) were found in lymphoid organs and these were mainly undivided. Comparable results were obtained on days 5 and 6, although OTII cell division proceeded over time (data not shown).

Activation of T cells leads to down-regulation of CD62L, allowing T cells to exit the LN and enter the circulation (21, 22). We studied CD62L expression on CD4<sup>+</sup> OTII cells that were transferred into B6.SJL mice on days 5 and 6 after immunization with peptide or PBS. Five days after immunization with OVA<sub>323-339</sub>, draining LN (Fig. 6A) and spleen (Fig. 6B) showed partial down-regulation of CD62L and its expression further decreased on day 6 (data not shown). After immunization with M-OVA<sub>323-339</sub>, similar results were obtained and the down-regulation of CD62L was even slightly accelerated. Sham immunization resulted in minimal CD62L down-regulation.

Blood samples were collected during the first week after immunization to monitor the circulation of OTII cells. Immunization with either peptide resulted in the reduced presence of OTII cells in the blood stream on day 2 as compared with sham immunization (Fig. 6C; 0.4 versus 0.8%). On days 4

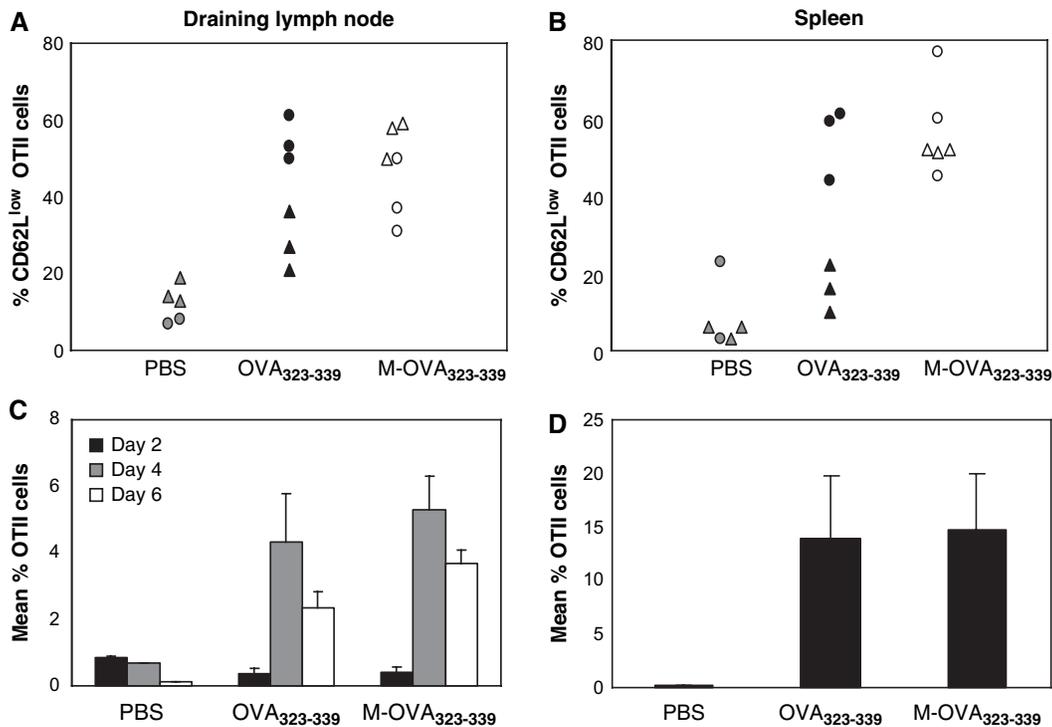
and 6, considerable numbers of OTII cells were present in the blood stream after immunization with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>, but no peptide-related differences were observed. The majority of circulating OTII cells went through at least six cell divisions and highly expressed CD62L (data not shown).

Circulation of OTII cells was also evaluated by their presence in peripheral tissue, such as the liver. As depicted in Fig. 6(D), similar numbers of OTII cells were isolated from the liver 4 days after immunization with either peptide, while no OTII cells were detectable after sham immunization. Isolation of OTII cells from the lung showed comparable results (data not shown).

Summarizing, these data indicate that mannosylated peptide induced normal clonal expansion and activation of OTII cells that were able to circulate through lymphoid as well as non-lymphoid tissue.

#### *Immunization with mannosylated peptide induces poor T<sub>H</sub>1 effector T cells*

We evaluated T cell effector functions also in congenic recipient mice of CFSE-labeled OTII cells by means of DTH responses to soluble OVA<sub>323-339</sub>, injected into the ear 3 days after immunization. As expected, immunization with OVA<sub>323-339</sub> in adjuvant induced functional effector T cells that elicited



**Fig. 6.** Similar recirculation of OTII cells after immunization with M-OVA<sub>323-339</sub>. CFSE-labeled OTII cells were transferred into B6.SJL mice and these were immunized 1 day later with PBS, OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>. Draining LN (A) and spleens (B) were isolated 5 (triangles) and 6 days (circles) after immunization and CD62L expression on CD4<sup>+</sup> CD45.2<sup>+</sup> OTII cells was analyzed (Filled symbols, OVA<sub>323-339</sub>; open symbols, M-OVA<sub>323-339</sub>; shaded symbols, PBS). Combined data from two experiments are shown. Blood samples were collected on days 2, 4 and 6 after immunization to monitor the presence of OTII cells. The numbers of CD45.2<sup>+</sup> OTII cells within the CD4<sup>+</sup> population were analyzed. Mean results ( $\pm$ SD) from at least three individual mice per time point are shown (C). Cells were isolated from the liver on day 4 and numbers of OTII cells within the live gate were analyzed; percentages of CD45.2<sup>+</sup> OTII cells within the CD4<sup>+</sup> population were calculated and mean data ( $\pm$ SD) from three individual mice are depicted (D).

a substantial DTH response ( $32 \pm 16\%$ ), as compared with sham-immunized mice (Fig. 7A,  $P < 0.01$ ). In contrast, immunization with M-OVA<sub>323-339</sub> in adjuvant induced a significantly lower DTH response ( $16 \pm 14\%$ ,  $P < 0.05$ ). The cell cycle plays an important role in T<sub>H</sub> cell differentiation and it has been described previously that IFN- $\gamma$  production is increased with each cell division (20). Therefore, we evaluated the *in vivo* production of IFN- $\gamma$  by CFSE-labeled OTII cells in the draining LN after transfer into congenic recipient mice 5 and 6 days after immunization. In response to immunization with OVA<sub>323-339</sub>, IFN- $\gamma$  production by CD4<sup>+</sup> OTII cells that went through multiple cell divisions was detectable. However, in M-OVA<sub>323-339</sub>-immunized mice, the IFN- $\gamma$  production by multiple-divided CD4<sup>+</sup> OTII cells hardly surpassed the level observed in sham-immunized mice (Fig. 7B and C).

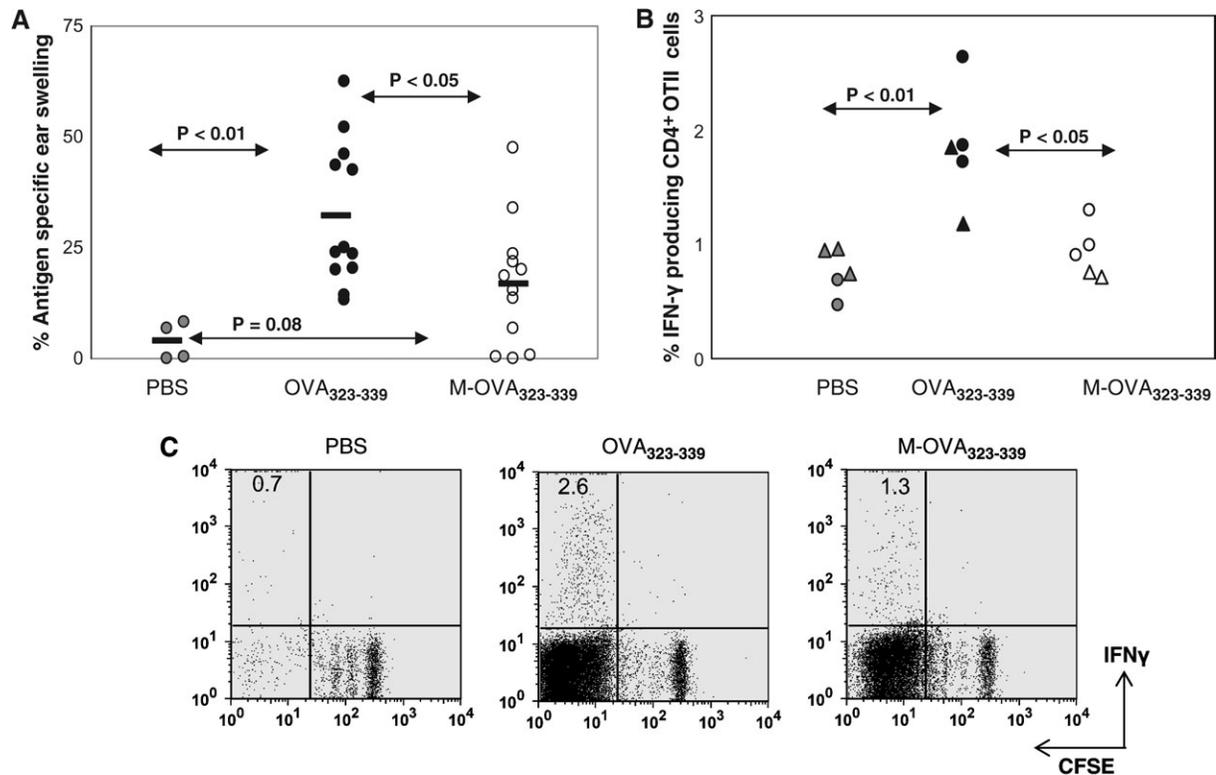
#### Local administration of soluble mannosylated peptide inhibits inflammation

Because immunization with M-OVA<sub>323-339</sub> in adjuvant induced poor DTH responses, we also characterized the cells that infiltrated the site of antigen challenge. For this purpose, C57BL/6 mice were reconstituted with OTII cells, immunized with OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> in adjuvant and subjected to an ear challenge with soluble OVA<sub>323-339</sub> on day 5. Mice that were immunized with M-OVA<sub>323-339</sub> showed significantly

decreased DTH responses as compared with mice that were immunized with OVA<sub>323-339</sub> (Fig. 8A,  $P < 0.05$ ). FACS analysis revealed that similar numbers of CD3<sup>+</sup> T cells were present in the ears of mice (data not shown), but that the absence of ear swelling was associated with a lower fraction of blast cells within this T cell population (Fig. 8B,  $P < 0.05$ ). As macrophages are considered indispensable effector cells in DTH responses, we analyzed the numbers of F4/80-positive cells and we observed that the numbers of macrophages were considerably, though not significantly, reduced (Fig. 8C).

To evaluate whether mannosylated peptide could trigger an inflammatory response mediated by preformed effector T cells, mice immunized with OVA<sub>323-339</sub> were also challenged with soluble M-OVA<sub>323-339</sub>. Importantly, ear swelling was significantly lower after injection of soluble M-OVA<sub>323-339</sub>, as compared with soluble OVA<sub>323-339</sub> ( $P < 0.01$ ). The reduced ear swelling was reflected by lower numbers of blastoid T cells (Fig. 8B,  $P < 0.001$ ) and macrophages (Fig. 8C,  $P < 0.05$ ). M-OVA<sub>323-339</sub>-immunized mice challenged with soluble M-OVA<sub>323-339</sub> showed the lowest DTH response of all groups (Fig. 8A,  $P = 0.06$  compared to challenge with OVA<sub>323-339</sub>), which was associated to reduced blastoid T cells and macrophages (Fig. 8B and C,  $P < 0.01$ ).

Thus, in addition to poor development of T<sub>H</sub>1 effector cells in response to immunization with M-OVA<sub>323-339</sub> in adjuvant,



**Fig. 7.** Immunization with M-OVA<sub>323-339</sub> induces poor effector T cells in congenic mice. CFSE-labeled OTII cells (CD45.2<sup>+</sup>) were transferred into B6.SJL mice (CD45.1<sup>+</sup>) and these were immunized 1 day later with PBS, OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub>. On day 3, OVA<sub>323-339</sub> was injected intradermally into the dorsal site of the ear and swelling was evaluated after 24 h (A). Combined data from three experiments are presented. Draining LN were isolated 5 (triangles) and 6 (circles) days after immunization and IFN- $\gamma$  production by the injected OTII cells was evaluated by flow cytometry, after gating for CD4<sup>+</sup> CD45.2<sup>+</sup> cells. The combined data from two experiments are presented (B). (C) A representative dot plot of each experimental group is depicted (gated on CD4<sup>+</sup> CD45.2<sup>+</sup>) to show that in particular multiple-divided OTII cells produce IFN- $\gamma$ .

local down-regulation of T<sub>H</sub>1 effector functions by soluble M-OVA<sub>323-339</sub> contributes to immune suppression.

## Discussion

We here show that immunization with M-OVA peptide results in immunological unresponsiveness of TCR transgenic OTII cells. Although the mannosylated peptide caused robust T cell proliferation *in vitro* and *in vivo*, immunized mice showed decreased DTH responses, diminished IgG2a levels in serum, reduced blastogenesis and minimal IFN- $\gamma$  production, all indicative for impaired T<sub>H</sub>1 cell effector functions. These data indicate that the proliferative cells did not develop full effector functions in response to mannosylated peptide or were unable to migrate into inflamed tissue. This finding is in line with previous studies, in which we have demonstrated that mannosylated self-peptide induced antigen-specific tolerance to EAE (7).

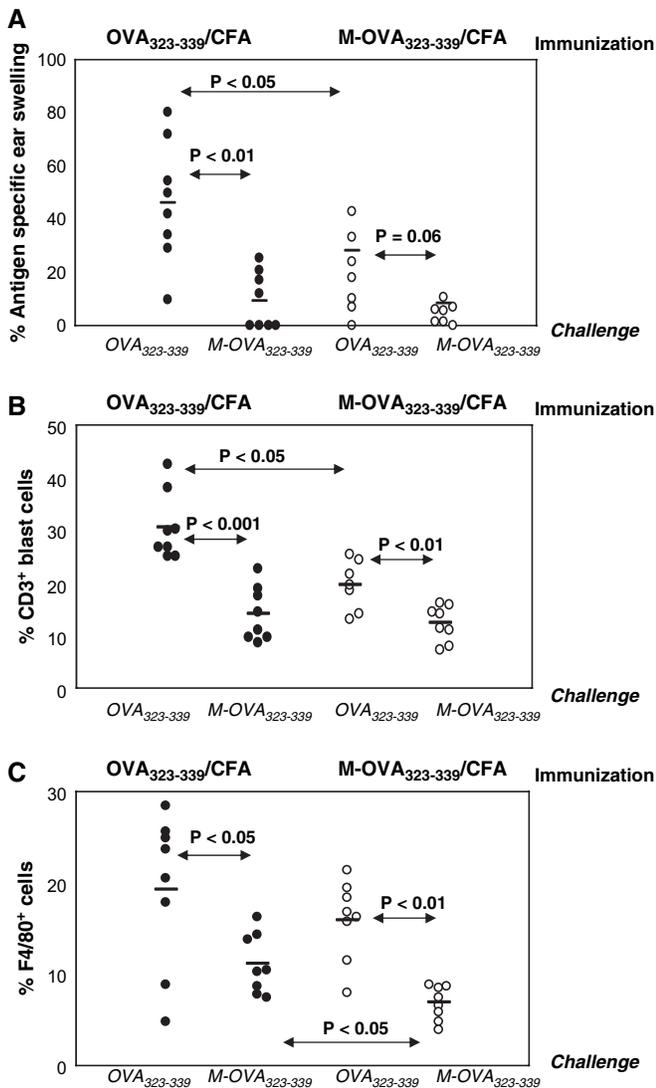
The immunological unresponsiveness did not result from poor recognition of the mannosylated peptide as *in vitro* stimulation of OTII and DO11.10 splenocytes with both peptides induced equal expansion of T cells with a T<sub>H</sub>1 phenotype (Fig. 1).

The possibility that immunization with M-OVA peptide caused a disturbed formation of memory T cells *in vivo* was addressed by monitoring the fate of CFSE-labeled OTII cells

and this approach revealed normal expansion of OTII cells, which seemed well activated based on the down-regulation of CD62L. Targeting of antigen toward DEC-205 induced enhanced early expansion of CD4<sup>+</sup> T cells, followed by immunological unresponsiveness due to deletion or the induction of CD5<sup>+</sup> T cells (8, 10). Therefore, it might be that major differences *in vivo* in response to mannosylated peptide occurred at earlier time points (before day 4), which have not been evaluated in the present studies.

Another possibility might be that effector T cells do not mediate their effects because they have impaired migratory capacities, as previously described by Mirenda *et al.* (23). However, OTII cells were detectable in peripheral organs as well as in the blood (Fig. 6). On the other hand, it has been described that only fully differentiated T<sub>H</sub>1 cells are able to infiltrate inflamed peripheral tissue, while non-polarized T cells are not (24, 25). Therefore, we cannot exclude that the differentiation state of OTII cells after immunization with M-OVA peptide limited their extravasation into local tissue during inflammation, resulting in poor DTH responses. Future studies will focus on the gene expression profile of T cells in response to mannosylated peptide to gain more insight into the mechanism of T cell unresponsiveness.

Besides the fact that M-OVA peptide induced poor T cell effector functions, pre-existing effector functions of fully polarized CD4<sup>+</sup> T were also inhibited by soluble M-OVA



**Fig. 8.** Local injection of soluble M-OVA<sub>323-339</sub> inhibits DTH responses. C57BL/6 mice were reconstituted with OTII cells and immunized with OVA<sub>323-339</sub> (filled circles) or M-OVA<sub>323-339</sub> (open circles) and on day 5, the *in vivo* recall response of T cells was tested by intra-dermal challenge with 12 nmol OVA<sub>323-339</sub> or M-OVA<sub>323-339</sub> into the ear. Ear thickness was measured 24 h later (A). Subsequently, the mice were sacrificed and the ears were collected to isolate the cells that accumulated in response to peptide injection. The numbers of blastoid cells within the CD3<sup>+</sup> T cell population (B) and the numbers of F4/80<sup>+</sup> macrophages (C) were determined by flow cytometry. The reduced swelling of ears was associated with significantly decreased numbers of T cell blasts and macrophages. Combined data from two experiments are presented and group means are indicated.

peptide. OVA<sub>323-339</sub>-immunized mice, with competent effector T cells, displayed impaired DTH responses to an intra-dermal ear challenge with soluble M-OVA peptide. These poor DTH responses were associated with reduced recruitment of T cell blasts and fewer infiltrating macrophages. This phenomenon cannot be explained by a lack of antigen presentation mediated by local APC, as co-culture experiments revealed presentation of M-OVA peptide in the ear (Fig. 4).

Therefore, it is likely that the cells presenting M-OVA peptide in the dermis or epidermis suppressed the effector functions of infiltrating T cells. In line with this, we have previously shown that treatment with soluble mannosylated self-peptide during ongoing autoimmunity can ameliorate clinical symptoms in EAE (26). Our findings extend previous observations by Higgins *et al.* (27) who described that CD4<sup>+</sup> effector T cells can be tolerated in lymphoid and non-lymphoid organs after injection of soluble antigen, resulting in abrogation of IFN- $\gamma$  and IL-2 production and reduced blastogenesis.

The presentation of mannosylated peptide *in vivo* itself was not impaired, but rather improved, according to the very efficient antigen presentation in the skin and draining LNs (Fig. 2). These results are in line with *in vitro* studies in which bis-mannosylated peptides were targeted to the mannose receptor, resulting in more efficient uptake and presentation of antigens (28–30). Although the exact nature of its receptor remains to be elucidated, it is likely that mannosylated peptides are targeted toward a member of the CLR family, which is widely expressed by APC in both lymphoid and non-lymphoid organs (31–33). It might well be that ligation of CLR by these bis-mannosylated peptides induces an anti-inflammatory program in APC. Although previous studies by Sheng *et al.* (34) indicated that cross-linking of CLR by mannan results in DC activation to some extent, this signal alone was insufficient for full DC maturation. It becomes increasingly clear that (pathogen mediated) signaling by CLR can modulate the immune response via the inhibition of pro-inflammatory nuclear factor kappa B-mediated signaling pathways (35–37). The existence of endogenous CLR ligands (38–40) is suggestive for self-control of immune responses; therefore, it might well be that our findings reflect physiological mechanisms that are indispensable for immune regulation and the prevention of autoimmunity.

Especially, immature APC are considered to be tolerogenic (41, 42) and Hawiger *et al.* (8, 9) showed that tolerance induction employing targeting of CLR was abrogated by ligation of CD40. Therefore, it is striking that M-OVA peptide induced a poor T<sub>H</sub>1 response despite the presence of complete adjuvant containing *M. tuberculosis*. Mannosylated peptides can thus be considered as a very powerful tool to control the effector functions of T cells.

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## Abbreviations

ATCC	American Type Culture Collection
APC	antigen-presenting cell
CLR	C-type lectin receptor
DTH	delayed-type hypersensitivity
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
LN	lymph node
M-OVA	mannosylated ovalbumin peptide
OVA	ovalbumin peptide

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