

The Efficacy of Immunotherapy in an Experimental Murine Model of Allergic Asthma Is Related to the Strength and Site of T Cell Activation During Immunotherapy¹

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In the present study, the relation between the efficacy of immunotherapy, and the strength and site of T cell activation during immunotherapy was evaluated. We used a model of allergic asthma in which OVA-sensitized and OVA-challenged mice display increased airway hyperresponsiveness, airway inflammation, and Th2 cytokine production by OVA-specific T cells. In this model, different immunotherapy strategies, including different routes of administration, or treatment with entire OVA or the immunodominant T cell epitope OVA_{323–339}, or treatment with a peptide analogue of OVA_{323–339} with altered T cell activation capacity were studied. To gain more insight in how immunotherapy affects allergen-specific T cells, the site of Ag-specific T cell activation and the magnitude of the T cell response induced during different immunotherapy strategies were determined using an adoptive transfer model. Our data suggest that amelioration of airway hyperresponsiveness and inflammation is associated with the induction of a strong, synchronized, and systemic T cell response, resulting in a decreased OVA-specific Th2 response. In contrast, deterioration of the disease after immunotherapy is associated with the induction of a weak nonsynchronized T cell response, resulting in the enhancement of the OVA-specific Th2 response after challenge. *The Journal of Immunology*, 2000, 165: 7207–7214.

Allergen immunotherapy, by s.c. injections of allergen extracts, is widely used for the treatment of insect venom and respiratory allergies (1, 2). Although this classical form of immunotherapy is beneficial for the treatment of rhinitis and insect venom allergy, it is less effective in allergic asthma (3–5). Moreover, immunotherapy can induce severe systemic reactions, such as asthma exacerbations and anaphylactic shock, due to cross-linking of allergen-specific IgE on mast cells (6). To circumvent the risk of cross-linking of allergen-specific IgE, the use of synthetic peptides containing immunodominant epitopes of allergens has been exploited. In several clinical trials, however, it has been shown that, although some patients showed a reduction of airway symptoms and Th2-associated responses, Fel d I peptide immunotherapy elicited allergic symptoms and late asthmatic reactions in cat-allergic asthmatics (7, 8). Moreover, we recently showed that in OVA-sensitized mice, s.c. immunotherapy with the immunodominant T cell epitope OVA_{323–339} enhanced both airway hyperresponsiveness (AHR)³ and eosinophilia upon OVA challenge (9), while an in vitro defined superagonistic analogue of this OVA_{323–339} epitope had a beneficial effect (10).

To date, little is known about how immunotherapy mediates its beneficial and adverse effects, and a prediction on the efficacy of immunotherapy is barely possible. Clinical studies suggested that immunotherapy exerted its beneficial effect by anergy induction of allergen-specific Th2 cells, or by modulation of the Th2 response toward a Th1 or a regulatory T cell response (11–13). These observations resulted in various hypotheses on the mechanism of allergen immunotherapy encompassing roles for the dose of Ag (including $t_{1/2}$ and number of ligands expressed on the APC), the affinity of the TCR for the MHC-peptide complex, the type of APC (differences in costimulatory molecules and cytokines), and the route of administration (10, 14–16).

In the present study, we used a murine model of allergic asthma, in which OVA-sensitized and OVA-challenged BALB/c mice display high levels of OVA-specific IgE Abs in serum, airway eosinophilia, AHR, and OVA-specific Th2 cells in lung tissue and lung-draining lymph nodes (LN), to investigate the relation between the efficacy of immunotherapy and T cell activation (17, 18). Immunotherapy was performed after sensitization before challenge, by s.c. or intranasal (i.n.) administration of OVA or the immunodominant epitope OVA_{323–339}. The outcome of these forms of immunotherapy was related to the site of Ag-specific T cell activation, and the magnitude of the T cell response using an adoptive T cell transfer model (19). In this model, limited numbers of fluorescent labeled (CFSE) OVA_{323–339}-specific DO11.10 T cells were transferred into naive BALB/c recipients, and T cell activation and division in various lymphoid organs were monitored at various days after immunotherapy with OVA, OVA_{323–339}, or the peptide analogue of OVA_{323–339}, OVA₃₃₆E-A, which we defined previously as a superagonistic peptide with comparable MHC-binding affinity as OVA_{323–339}, but more potent in the induction of T cell proliferation and Th1 cytokine induction in DO11.10 T cells in vitro (10).

Based on the present results, we postulate that the efficacy of immunotherapy is dependent on the strength and site of T cell activation during immunotherapy.

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³ Abbreviations used in this paper: AHR, airway hyperresponsiveness; i.n., intranasal; LN, lymph node; MCh, methacholine; BAL, bronchoalveolar lavage.

Materials and Methods

Animals

Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Specified pathogen-free male BALB/c mice (6–8 wk) and OVA_{323–339} TCR transgenic DO11.10 mice on a BALB/c background (20) were bred at the Central Animal Laboratory (Utrecht, The Netherlands), and housed in macrolon cages and provided with OVA-free food and water ad libitum.

Peptides and proteins

OVA (grade V) was obtained from Sigma (St. Louis, MO), and OVA_{323–339} peptide (ISQAVHAAHAEINEAGR) was synthesized by Isogen (Isogen Bioscience, Maarn, The Netherlands). The peptide analogue OVA₃₃₆E-A, in which the glutamic acid at the TCR contact residue at position 336 was substituted into alanine, was synthesized by automatic multiple peptide synthesis (21). Peptides were analyzed and purified by reversed-phased HPLC, and checked by fast atom bombardment mass spectrometry.

Isolation and labeling of OVA_{323–339}-specific T cells

OVA_{323–339}-specific DO11.10 T cells were obtained from TCR transgenic mice by negative selection, as described previously (10). Cells obtained after depletion were shown to be >90% OVA_{323–339}-specific T cells, as demonstrated by FACS analysis using the clonotype-specific Ab KJ1.26 (22). DO11.10 T cells were resuspended at a density of 2×10^7 cells/ml in PBS, and labeled with carboxyl-fluorescein diacetate succinimidyl ester (CFDASE; Molecular Probes, Eugene, OR) (23) in a final concentration of 0.5 μ M for 10 min at room temperature. Unbound CFDASE or the deacetylated form CFSE was quenched by the addition of an equal volume of heat-inactivated FCS. Analysis of cells immediately following CFSE labeling indicated a labeling efficiency of >99%, and a T cell viability of >95%. T cells were homogeneously labeled, and in vitro studies demonstrated that the labeling remained stable for at least 10 days in vitro (data not shown).

Determination of in vivo T cell activation

A total of 1×10^7 CFSE-labeled DO11.10 T cells was administered i.v. into naive BALB/c mice, which resulted in a homogenous distribution of labeled DO11.10 T cells in various lymphoid tissues and blood. Approximately 1% of the Thy-1.2⁺ population consisted of DO11.10 T cells, and a time course study showed that the relative and absolute number of DO11.10 T cells did not change within 72 h after transfer (data not shown). Mice were challenged s.c. or i.n. with PBS, OVA, OVA_{323–339}, or OVA₃₃₆E-A (300 μ g in 50 and 10 μ l, respectively). After 1, 2, or 3 days, mice were sacrificed, and mandibular, axillary/brachial, popliteal, lung-draining, and mesenteric LN, and spleens and blood were collected. Single cell suspensions were prepared and incubated with Abs against Fc γ R2/III (24G2) to block a specific binding. Subsequently, cells were incubated with a PE-coupled Ab to Thy-1.2 (PharMingen, San Diego, CA; 1:100). After staining, erythrocytes were lysed by FACS lysing solution (Becton Dickinson, San Jose, CA). To exclude dead cells from analysis, propidium iodide was added directly before each measurement. Per sample, 2×10^6 Thy-1.2⁺ events were measured by a FACScalibur, and the CFSE fluo-

rescence intensity was determined using CellQuest (Becton Dickinson). Because CFSE segregates equally between daughter cells upon cell division, the number of fluorescent peaks (as determined by CFSE intensity) reflects the number of mitotic events.

Induction of experimental asthma and treatment protocols

Previously, we described the development of an OVA-based murine model with features reminiscent of allergic asthma (17). Briefly, BALB/c mice were sensitized by seven i.p. injections of 10 μ g OVA in 0.5 ml pyrogen-free saline without adjuvant on alternate days. Two weeks later, mice were exposed to OVA (2 mg/ml) or saline aerosol challenges for 5 min on 8 consecutive days. Aerosols were performed in a plexiglass exposure chamber coupled to a Jet nebulizer (Pari IS-2 Jet nebulizer; PARI Respiratory Equipment, Richmond, VA; particle size 2–3 μ m) driven by compressed air at a flow rate of 6 L/min. Twenty-four hours after the last challenge airway responsiveness to methacholine (MCh) was measured in vivo, and the infiltration of inflammatory cells in the bronchoalveolar lavage (BAL) was determined (9, 18). Subcutaneous treatments were performed 14 and 17 days after the last sensitization, by administration of OVA, OVA_{323–339}, or PBS (see Table I for final dose). Intranasal treatments were performed 14, 16, and 18 days after the last sensitization, by administration of OVA, OVA_{323–339}, or PBS (see Table II for final dose). Animals were either sacrificed 24 h after the last i.n. treatment, or challenged 7 days after the last treatment, as described above.

Cytokine analysis of lung-draining LN cells

Twenty-four hours after the last aerosol, lung-draining LN were collected, and single cell suspensions were made. Cells (2×10^5 cells/well in 96-well plates) were cultured in IMDM supplemented with 10% FCS, 2 nM L-glutamine, 100 endotoxin units penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-ME, in the presence or absence of OVA (10 μ g/ml). After 120 h of culture, the disease-associated cytokines IL-4 and IL-5 in the supernatants were determined by sandwich ELISA (PharMingen). All LN cultures showed comparable T cell cytokine production after anti-CD3 stimulation (data not shown).

Data analysis

Unless stated otherwise, data are expressed as mean $\pm$ SEM, and evaluated using an ANOVA, followed by a Dunnett test for comparison between groups. A probability value of $p < 0.05$ was considered statistically significant.

Results

The route of Ag administration affects the efficacy of immunotherapy of allergen-induced airway manifestations

To determine the efficacy of immunotherapy after different Ag doses and different administration routes, BALB/c mice were sensitized with OVA and treated s.c. or i.n. with different doses of OVA or the immunodominant epitope OVA_{323–339} (Tables I and II). Subsequently, mice were challenged with OVA or saline, and

Table I. Allergen-induced airway manifestations and OVA-specific Th2 responses after s.c. treatment with OVA or OVA_{323–339}^a

Sensitization	Treatment (total concentration s.c.)	Challenge	AHR MCh (250 μ g/kg)	Eosinophils ($\times 10^3$)	OVA-Specific IL-4	OVA-Specific IL-5
OVA	PBS	Saline	2.1 $\pm$ 1.1	–	–	–
OVA	PBS	OVA	61.3 $\pm$ 8.3*	67 $\pm$ 23*	++	++
OVA	OVA (0.3 mg)	Saline	2.4 $\pm$ 1.6	–	–	–
OVA	OVA (0.3 mg)	OVA	8.9 $\pm$ 3.4*#	20 $\pm$ 2*#	↓↓#	↓↓#
OVA	OVA (3.0 mg)	Saline	2.2 $\pm$ 1.3	–	–	–
OVA	OVA (3.0 mg)	OVA	8.4 $\pm$ 2.5*#	11 $\pm$ 8*#	↓↓#	↓↓#
OVA	OVA _{323–339} (0.3 mg)	Saline	1.8 $\pm$ 1.3	–	–	–
OVA	OVA _{323–339} (0.3 mg)	OVA	119.6 $\pm$ 24.3*#	154 $\pm$ 29*#	↑/=	↑/=
OVA	OVA _{323–339} (3.0 mg)	Saline	2.2 $\pm$ 1.5	–	–	–
OVA	OVA _{323–339} (3.0 mg)	OVA	127.8 $\pm$ 10.5*#	128 $\pm$ 34*#	↑/=	↑/=

^a BALB/c mice were sensitized as described in *Materials and Methods*. Subsequently, mice were treated s.c. with PBS, OVA, or OVA_{323–339}. Seven days later mice were challenged with OVA or saline, and AHR to i.v. administered MCh (AHR in cmH₂O/ml/s) and the absolute number of eosinophils in the BAL were determined. Lung-draining LN were isolated, and cells were cultured in the presence of OVA to determine the production of disease-associated cytokines by OVA-specific T cells. Not detectable; +, clearly detectable; ↑/↓, increase/decrease compared with PBS-treated mice of comparable challenge; =, comparable with PBS-treated mice of comparable challenge. *, $p < 0.05$ compared with saline-challenged mice from comparable treatment groups. #, $p < 0.05$ compared with PBS-treated OVA-challenged mice.

Table II. Allergen-induced airway manifestations and OVA-specific Th2 responses after i.n. treatment with OVA or OVA₃₂₃₋₃₃₉^a

Sensitization	Treatment (total concentration s.c.)	Challenge	AHR MCh (250 µg/kg)	Eosinophils (×10 ³)	OVA-Specific IL-4	OVA-Specific IL-5
OVA	PBS		2.3 ± 1.0	0	—	—
OVA	PBS	Saline	2.9 ± 1.1	0	—	—
OVA	PBS	OVA	32.1 ± 6.3*	82 ± 16*	++	++
OVA	OVA (0.3 mg)		58.3 ± 9.7 [†]	69 ± 12 [†]	↑ [†]	↑ [†]
OVA	OVA (3.0 mg)		NT ^b	219 ± 43 [†]	↑↑#	↑↑#
OVA	OVA (0.3 mg)	Saline	3.6 ± 1.9	9 ± 4*	↑ [†]	↑ [†]
OVA	OVA (0.3 mg)	OVA	98.2 ± 21.4*#	166 ± 17*#	↑↑#	↑↑#
OVA	OVA ₃₂₃₋₃₃₉ (0.3 mg)		NT	0	—	—
OVA	OVA ₃₂₃₋₃₃₉ (0.3 mg)	Saline	2.6 ± 1.2	0	—	—
OVA	OVA ₃₂₃₋₃₃₉ (0.3 mg)	OVA	34.9 ± 5.9*	69 ± 11*	=	=

^a BALB/c mice were sensitized as described in *Materials and Methods*. Subsequently, mice were treated i.n. with PBS, OVA, or OVA₃₂₃₋₃₃₉. Seven days later, mice were challenged with OVA or saline, and AHR to i.v. administered MCh (AHR in cmH₂O/ml/s) and the absolute number of eosinophils in the BAL were determined. Lung-draining LN were isolated, and cells were cultured in the presence of OVA to determine the production of disease-associated cytokines by OVA-specific T cells. Not detectable; +, clearly detectable; ↑/↓, increase/decrease compared with PBS-treated mice of comparable challenge; =, comparable with PBS-treated mice of comparable challenge. *, *p* < 0.05 compared with saline-challenged mice from comparable treatment groups. #, *p* < 0.05 compared with PBS-treated OVA-challenged mice. †, *p* < 0.05 compared with PBS-treated nonchallenged mice.

^b NT, Not tested.

AHR to MCh, airway eosinophilia, and OVA-specific Th2 responses was determined. In all treatment groups, OVA challenge induced a significant increase in the airway responsiveness to MCh and airway eosinophilia compared with the corresponding saline-challenged groups. Subcutaneous administration of OVA significantly reduced AHR, eosinophilia, and OVA-specific Th2 responses in OVA-challenged mice, irrespective of the dose of OVA used for immunotherapy (Table I). In contrast for both doses tested, s.c. administration of OVA₃₂₃₋₃₃₉ significantly enhanced the airway manifestations (Table I). These data show that irrespective of the dose used, s.c. immunotherapy with OVA ameliorated the disease process, while s.c. immunotherapy with OVA₃₂₃₋₃₃₉ deteriorated the disease process. In contrast to s.c. immunotherapy, i.n. administration of OVA deteriorated airway manifestations. Already 24 h after the last i.n. OVA administration before OVA challenge, AHR and eosinophilia could be detected (data not shown). After OVA challenge, mice showed significantly increased AHR and Th2 cytokine production compared with PBS-treated mice (Table II). Interestingly, i.n. OVA₃₂₃₋₃₃₉ administration had no effect on the development of airway symptoms (Table II). These data suggest that primarily the Ag composition and route determine the effectiveness of immunotherapy.

To analyze where T cell activation occurred in vivo after s.c. or i.n. administration of Ag, CFSE-labeled DO11.10 T cells were administered i.v. in naive BALB/c mice. Subsequently, mice were treated s.c. or i.n. with PBS, OVA, or OVA₃₂₃₋₃₃₉ (300 µg), and DO11.10 T cell proliferation in different lymphoid tissues was determined by FACS analysis at various time points.

Analysis of the cell numbers and division cycles of DO11.10 T cells in blood after s.c. and i.n. Ag administration

Subcutaneous administration of PBS had no effect on the number of DO11.10 T cells circulating in the blood, and a time course (0–72 h) showed that the relative and absolute number of DO11.10 T cells did not change in the first 72 h after transfer (Fig. 1A, and data not shown). In contrast, s.c. administration of OVA resulted in a >75% reduction of DO11.10 T cells in the blood within 24 h, which lasted until 48 h after OVA administration. The remaining cells in the blood displayed the fluorescence intensity that corresponded with undivided cells. Seventy-two hours after s.c. OVA administration, the population of DO11.10 T cells in the blood had strongly increased and consisted largely (>80%) of T cells that displayed a CFSE fluorescence intensity of cells that had divided four or more times (Fig. 1, B and C). Subcutaneous administration

of OVA₃₂₃₋₃₃₉ resulted also in mobilization of DO11.10 T cells from the blood, but this process was slower than after OVA administration (Fig. 1A). After 24 h, ~35%, and after 48 h, 65% of the DO11.10 T cells had disappeared from the blood. After 72 h, the population of DO11.10 T cells in the blood had slightly increased, but the population of DO11.10 T consisted merely (>85%) of cells that had not or only once divided (Fig. 1, B and C). Similar to s.c. OVA₃₂₃₋₃₃₉ administration, i.n. OVA administration resulted in a slow mobilization of DO11.10 T cells from the blood and low numbers of divided cells 72 h after i.n. OVA administration. Intranasal OVA₃₂₃₋₃₃₉ administration mobilized only a very small proportion of the DO11.10 T cell population from the blood (<25%), and after 72 h, all DO11.10 T cells still displayed the fluorescence intensity of undivided cells (Fig. 1, B and C).

Analysis of the cell numbers and division cycles of DO11.10 T cells in draining LN after s.c. and i.n. Ag administration

Subcutaneous or intranasal administration of PBS did not induce an increase in the number of DO11.10 T cells in brachial or mandibular LN (LN that drain the site of, respectively, s.c. and i.n. Ag administration) (Fig. 2A). Subcutaneous administration of OVA resulted in a 6-fold increase of DO11.10 T cells in the brachial LN after 72 h. Analysis of DO11.10 T cell division showed that already 48 h after s.c. OVA administration, >90% of the DO11.10 T cells in the brachial LN had divided and displayed fluorescence intensities that correlated with the intensities after four and five cell divisions (Fig. 2, B and C). The DO11.10 T cells in the brachial LN continued dividing, and 72 h after s.c. administration of OVA, >65% of the T cells displayed a fluorescence that correlated with the intensities after six to seven cell divisions (Fig. 2, B and C). In contrast, 48 h after s.c. OVA₃₂₃₋₃₃₉ administration, only 20% of the DO11.10 T cells displayed a fluorescence intensity that correlated with one to three cell divisions. Seventy-two hours after s.c. OVA₃₂₃₋₃₃₉ administration, >85% of the DO11.10 T cells displayed fluorescence intensities that corresponded with one to six cell divisions (Fig. 2, B and C). Interestingly, both i.n. OVA and i.n. OVA₃₂₃₋₃₃₉ administration resulted in comparable increase of the DO11.10 T cell population in the mandibular LN. After 48 h, ~30% of the DO11.10 T cells displayed fluorescence intensities correlating with one to four cell divisions, while after 72 h, ~85% of the DO11.10 T cells displayed fluorescence intensities that corresponded with one to six cell divisions (Fig. 2, B and C).

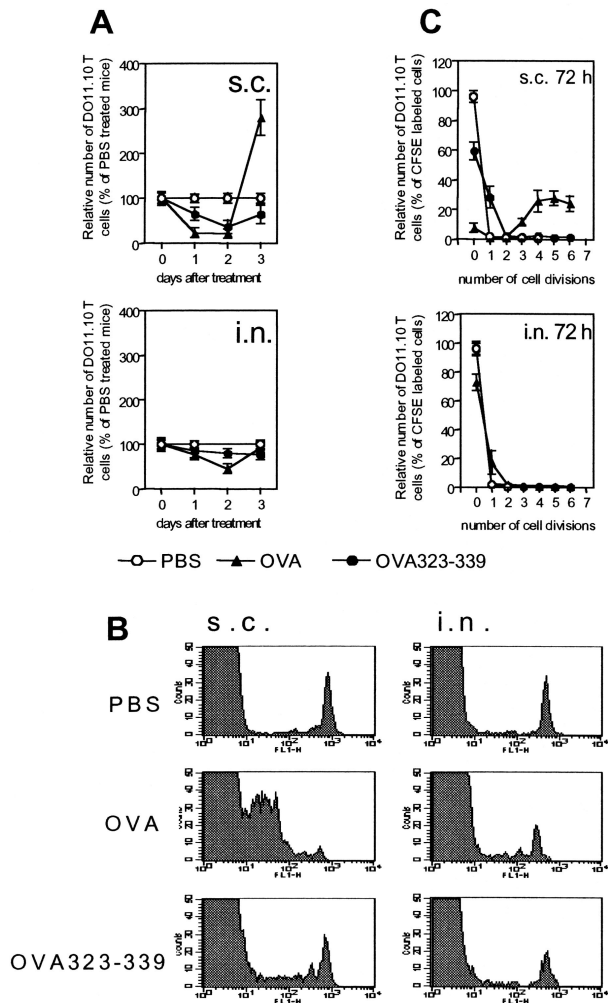


FIGURE 1. Analysis of DO11.10 T cells in blood after s.c. or i.n. Ag administration. A total of 1×10^7 CFSE-labeled DO11.10 T cells was injected i.v. in naive BALB/c mice. Subsequently, mice were treated s.c. or i.n. with PBS, OVA, or OVA₃₂₃₋₃₃₉. After 24, 48, and 72 h, the DO11.10 T cell population in blood was analyzed by FACS. **A**, Relative numbers of DO11.10 T cells in the blood compared with PBS-treated mice at 24, 48, and 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group). **B**, CFSE-labeled DO11.10 T cells in the blood 72 h after s.c. or i.n. administration. Undivided DO11.10 T cells display the maximal cellular fluorescence intensity, which is sequentially halved with each successive T cell division. Figures show representative results from one mouse (one of six). **C**, Number of cell divisions of CFSE-labeled DO11.10 T cells 72 h after treatment, as calculated by the halving of the FL1 signal after each successive cell division. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group).

Analysis of the cell numbers and division cycles of DO11.10 T cells in the spleen after s.c. and i.n. Ag administration

Subcutaneous administration of OVA resulted in a strong increase of the population of DO11.10 T cells in the spleen. Analysis showed that $>90\%$ of the DO11.10 T cells displayed fluorescence intensities of cells that had divided three to five times within 48 h, and that after 72 h, $>65\%$ of the T cells displayed fluorescence intensities of cells that had divided six to seven times (Fig. 3, *B* and *C*). In contrast, 48 h after s.c. OVA₃₂₃₋₃₃₉ administration, only 20% of the DO11.10 T cells displayed fluorescence intensities of cells that had divided one to three times, while after 72 h, $>75\%$ of the DO11.10 T cells displayed intensities that correlated with

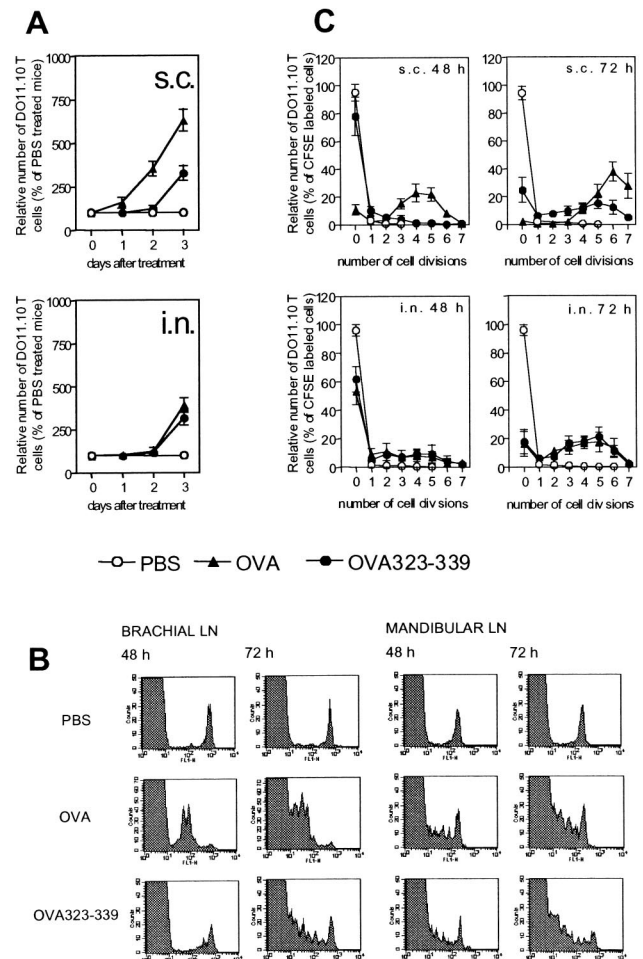


FIGURE 2. Analysis of DO11.10 T cells in the draining LN after s.c. or i.n. Ag administration. **A**, Relative numbers of DO11.10 T cells in the LN draining the site of Ag administration. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group). **B**, CFSE-labeled DO11.10 T cells in the draining LN 48 and 72 h after treatment. Figures show representative results from one mouse (one of six). **C**, Number of cell divisions of CFSE-labeled DO11.10 T cells 48 and 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group).

one to six cell divisions (Fig. 3, *B* and *C*). Intranasal administration of OVA resulted in comparable DO11.10 T cell division, as observed after s.c. OVA₃₂₃₋₃₃₉ administration, while after i.n. OVA₃₂₃₋₃₃₉ administration, all DO11.10 T cells displayed the fluorescence intensity of undivided cells (Fig. 3, *B* and *C*).

In summary, s.c. administration of OVA, which has a beneficial effect on airway manifestations and reduces OVA-specific Th2 responses, resulted in a strong, rapid, and synchronized T cell response, both local as well as systemic. In contrast, both s.c. OVA₃₂₃₋₃₃₉ and i.n. OVA administration resulted in a slower induced and less synchronized local T cell response, and only a weak systemic response. Interestingly, both s.c. OVA₃₂₃₋₃₃₉ and i.n. OVA administration aggravated airway responsiveness and inflammation. Intranasal OVA₃₂₃₋₃₃₉ administration induced only a nonsynchronized local response, and had no effect on the disease. These findings suggest that beneficial effects of therapy coincide with a rapid, strong, and synchronized systemic T cell activation, while adverse effects occur after weak nonsynchronized T cell activation.

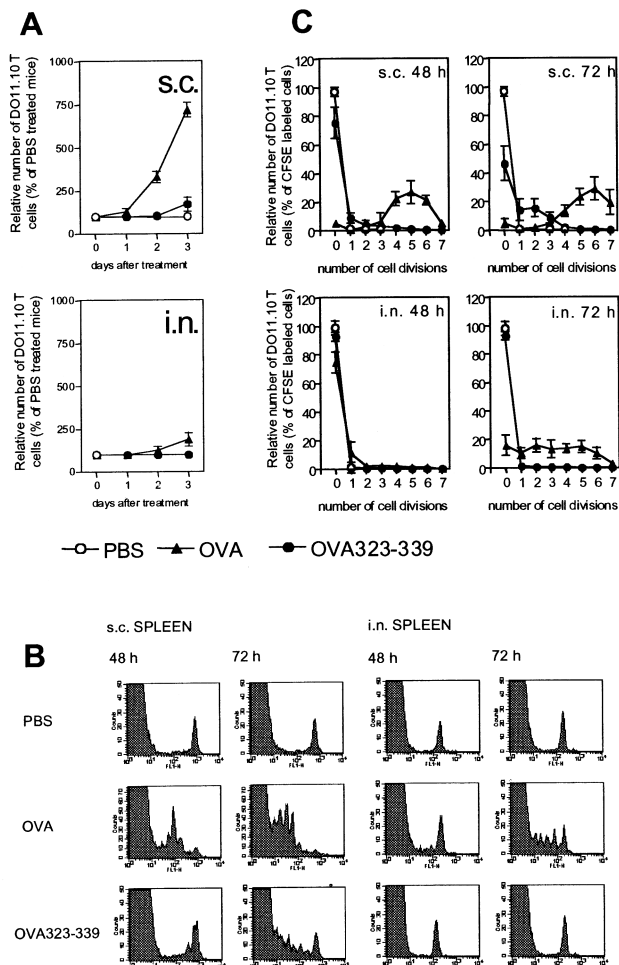


FIGURE 3. Analysis of DO11.10 T cells in the spleen after s.c. or i.n. Ag administration. *A*, Relative numbers of DO11.10 T cells in the spleen. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group). *B*, CFSE-labeled DO11.10 T cells in the spleen 48 and 72 h after treatment. Figures show representative results from one mouse (one of six). *C*, Number of cell divisions of CFSE-labeled DO11.10 T cells 48 and 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 6$ mice per group).

Effect of s.c. administration of a peptide analogue of OVA₃₂₃₋₃₃₉ on DO11.10 T cell responses in blood and lymphoid organs

To test this hypothesis, we analyzed T cell mobilization and activation after s.c. administration of a peptide analogue of OVA₃₂₃₋₃₃₉, OVA₃₃₆E-A. Recently, we demonstrated that peptide analogue OVA₃₃₆E-A has a comparable MHC class II-binding affinity as OVA₃₂₃₋₃₃₉, but is a more potent inducer of T cell proliferation and Th1-associated cytokine production in DO11.10 T cells in vitro (10). Furthermore, we showed that, in contrast with the wild-type OVA₃₂₃₋₃₃₉ peptide, s.c. treatment with 300 μ g OVA₃₃₆E-A in OVA-sensitized mice markedly reduced airway inflammation and Th2 cytokine production by OVA-specific T cells upon OVA challenge (10).

We show in this study that s.c. administration of OVA or OVA₃₃₆E-A both resulted in a comparable mobilization of DO11.10 T cells from the blood, and 48 h after s.c. administration, >70% of the DO11.10 T cells had disappeared (Fig. 4*A*). After 72 h, the population of DO11.10 T cells in the blood had strongly increased, and consisted merely of T cells displaying a CFSE fluorescence intensity that correlated with cells that had divided three or more times (Fig. 4, *B* and *C*). As also shown in Fig. 1, s.c. OVA₃₂₃₋₃₃₉ administration resulted in a slower mobilization of

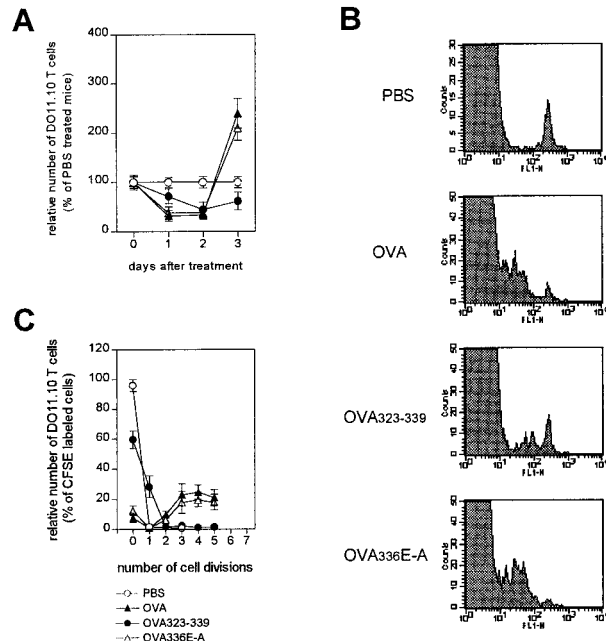


FIGURE 4. Analysis of DO11.10 T cells in blood after s.c. Ag administration. *A*, Relative numbers of DO11.10 T cells in blood. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group). *B*, CFSE-labeled DO11.10 T cells in blood 72 h after treatment. Figures show representative results from one mouse (one of five). *C*, Number of cell divisions of CFSE-labeled DO11.10 T cells 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group).

DO11.10 T cells from the blood, and after 72 h, the DO11.10 T cell population in the blood consisted mainly of cells that had not or only once divided (Fig. 4, *B* and *C*).

Furthermore, s.c. administration of OVA or OVA₃₃₆E-A both resulted in a comparable strong synchronized proliferative response and increase in DO11.10 T cells in the draining brachial LN (Fig. 5*A*). Within 48 h, >70% of the DO11.10 T cells displayed a fluorescence intensity of cells that had divided two to four times, whereas after 72 h, 90% of the DO11.10 T cells displayed a fluorescence intensity of cells that had divided four or more times (Fig. 5, *B* and *C*). As observed before, s.c. OVA₃₂₃₋₃₃₉ administration resulted in a weaker and less synchronized response; after 72 h, 70% of the DO11.10 T cells displayed a fluorescence intensity that correlated with one to seven cell divisions (Fig. 5, *B* and *C*). Comparable results were found in the spleen. Subcutaneous OVA or OVA₃₃₆E-A administration both resulted in a strong rapid and synchronized cell proliferation in >80% of the DO11.10 T cells within 48 h, while s.c. OVA₃₂₃₋₃₃₉ administration resulted in a weaker and less synchronized response (Fig. 6, *B* and *C*).

Discussion

In the present study, we evaluated the relationship between the efficacy of different immunotherapy strategies, and the strength and location of T cell activation during immunotherapy in a murine model of allergic asthma. Previously, we demonstrated that s.c. treatment of OVA-sensitized mice with OVA or a peptide analogue OVA₃₃₆E-A resulted in a decrease in AHR, airway inflammation, and cytokine production by OVA-specific Th2 cells, while s.c. treatment with the wild-type T cell epitope OVA₃₂₃₋₃₃₉ aggravated airway manifestations (9, 10). We show in this study that these effects were primarily associated with the Ag composition, protein vs peptide, and were independent of the dose used for

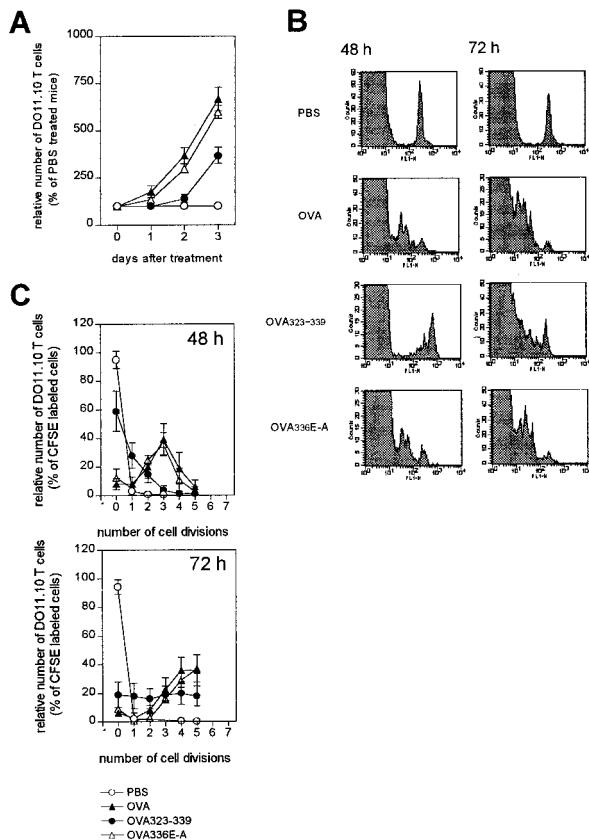


FIGURE 5. Analysis of DO11.10 T cells in the draining LN after s.c. Ag administration. **A**, Relative numbers of DO11.10 T cells in the brachial and axillary LN. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group). **B**, CFSE-labeled DO11.10 T cells in the LN 48 and 72 h after treatment. Figures show representative results from one mouse (one of five). **C**, Number of cell divisions of CFSE-labeled DO11.10 T cells 48 and 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group).

immunotherapy. Furthermore, the route of administration seems to be of importance, because in contrast to s.c. immunotherapy, i.n. OVA immunotherapy aggravated airway manifestations, while i.n. treatment with OVA₃₂₃₋₃₃₉ had no effect on disease development.

To gain more insight how immunotherapy affects allergen-specific T cell responses, we studied the site and strength of Ag-specific T cells activation after s.c. and i.n. administration of OVA, OVA₃₂₃₋₃₃₉, or peptide analogue OVA_{336E-A} in a transfer model with CFSE-labeled OVA₃₂₃₋₃₃₉-specific DO11.10 T cells. Subcutaneous administration of OVA or OVA_{336E-A} resulted in a rapid mobilization of almost all fluorescent DO11.10 T cells from the blood, which lasted more than 48 h. The DO11.10 T cells in the draining LN as well as in the spleen divided vigorously and synchronic within 48 h, suggesting that T cell activation was initiated in these organs, and not due to migration of divided cells from the draining LN via the blood to the spleen. In contrast, s.c. OVA₃₂₃₋₃₃₉ and i.n. OVA administration mobilized only a part of the DO11.10 T cell population in the blood, and resulted in a slower induced and nonsynchronic T cell division in the draining LN. Remarkably, only marginal cell division in the spleen was observed within 48 h. After 72 h, most DO11.10 T cells in the spleen displayed fluorescence levels of divided cells. This was most likely due to migration of T cells that were activated in the draining LN, because at 72 h, high numbers of divided DO11.10 T cells were observed in the blood as well. Intranasal administration

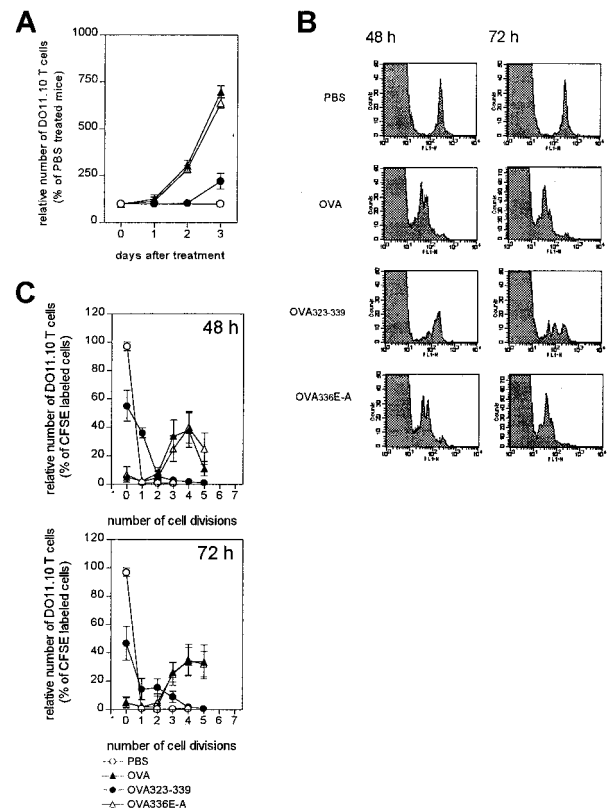


FIGURE 6. Analysis of DO11.10 T cells in the spleen after s.c. Ag administration. **A**, Relative numbers of DO11.10 T cells in the spleen. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group). **B**, CFSE-labeled DO11.10 T cells in the spleen 48 and 72 h after treatment. Figures show representative results from one mouse (one of five). **C**, Number of cell divisions of CFSE-labeled DO11.10 T cells 48 and 72 h after treatment. Data are expressed as mean $\pm$ SEM ($n = 5$ mice per group).

of OVA₃₂₃₋₃₃₉ resulted hardly in mobilization of DO11.10 T cells from the blood, although T cell division in the draining LN was comparable with i.n. OVA administration. These findings indicate that the absence of modulatory effects on airway symptoms after i.n. OVA₃₂₃₋₃₃₉ therapy was not due to rapid degradation of the peptide or lack of T cell activation. However, in contrast to i.n. OVA administration, no T cell division in the spleen was observed after i.n. OVA₃₂₃₋₃₃₉ administration.

All together these data suggest that amelioration of AHR, eosinophilia, and a decreased OVA-specific Th2 response are associated with the induction of a strong, synchronized, and systemic T cell response during immunotherapy. In contrast, deterioration of the disease and an increase of the OVA-specific Th2 response are associated with the induction of a weak nonsynchronic T cell response in the immunotherapy draining LN. These findings are consistent with the findings of Kearney et al. (19), who demonstrated that systemic administration (by i.v. injection) of OVA or OVA₃₂₃₋₃₃₉ in this T cell transfer model induced a rapid, strong, transient DO11.10 T cell proliferation, resulting in a state of non-responsiveness of these T cells upon subsequent challenge in vivo or in vitro. Moreover, they described that the induction of a more local and less transient response, by s.c. administration of OVA₃₂₃₋₃₃₉ in CFA, resulted in a significantly enhanced T cell response upon in vitro stimulation with OVA₃₂₃₋₃₃₉ (19, 24). Previously, we described that successful immunotherapy was associated with decreased Th2 responses (9, 10). Because we did not observe a shift to another cytokine profile, it could well be possible

that nonresponsiveness or apoptosis of OVA-specific T cells was induced. Whether in our model OVA-specific T cells actually become nonresponsive after successful immunotherapy is currently under investigation.

The most prevailing hypotheses on the mechanism of immunotherapy state that immunotherapy induces anergy, or a Th1 phenotype in allergen-specific Th2 cells. Because beneficial effects of immunotherapy are observed after treatment with relative high doses of allergen, the availability of the Ag, including the dose and $t_{1/2}$ of the Ag, the number of Ag-MHC complexes on the APC, and the affinity for the MHC and TCR have been suggested to play an important role in the efficacy of immunotherapy (13–16).

Several in vitro and in vivo studies demonstrated that high Ag doses or ligands with high MHC-binding affinity, both resulting in high ligand densities on APC, can induce hyporesponsiveness or Th1 phenotypic cells, whereas low ligand densities are associated with the induction of Th2 responses (25–28). However, our studies suggest that merely differences in the number of MHC-peptide complexes cannot explain the opposite immunotherapeutic effects.

Alternatively, the APC type presenting the allergen during immunotherapy may play a crucial role in the modulation of the allergen-specific Th2 response. The differences in T cell responses might be due to differences in the nature and costimulatory capacities of the APC involved (29, 30). Because OVA has to be processed by professional APC before it can be presented to T cells, while OVA_{323–339} can bind exogenously to MHC class II (31), we cannot fully exclude that OVA and OVA_{323–339} are presented by different APC types, or caused a different activation state in the APC presenting the ligand. However, s.c. treatment with the OVA₃₃₆-E-A peptide analogue resulted in a comparable response as treatment with entire OVA. Because it is not likely that after s.c. administration of peptides with comparable length and MHC-binding affinity these peptides are presented by different APC types (32), these findings suggest that besides the APC type, other factors play a crucial role in the modulation of the disease and the induction of a strong systemic response. Another explanation involves the affinity of the TCR for the MHC-peptide complex. Kinetic models of TCR-MHC interaction describe a relationship between T cell activation and the affinity of the TCR for its ligand (33, 34). Expression of high TCR affinity ligands is associated with a prolonged interaction with the TCR, which leads to the induction of a Th1 phenotype, anergy, or apoptosis, whereas low affinity ligands are associated with the induction of a Th2 phenotype and T cell anergy (35–37). Our finding that treatment with OVA_{323–339} resulted in a weak systemic T cell response, and an increase of the Th2 response, whereas treatment with the OVA₃₃₆-E-A peptide analogue induced a strong systemic T cell response and inhibited the Th2 response, could well be due to a higher TCR-binding affinity of the MHC-OVA₃₃₆-E-A complex. Altered TCR affinity may also explain the differences observed between OVA and OVA_{323–339} treatments. It has been demonstrated recently that OVA_{323–339} can bind to the MHC in different configurations, leading to altered TCR exposed residues (38). Processing of OVA by APC may favor a specific peptide orientation in the MHC, due to the presence of OVA_{323–339} flanking residues, with relative higher affinity for the TCR than the synthetic OVA_{323–339} (39).

Based on our data, we postulate that the strength of systemic T cell activation during immunotherapy is crucial for the beneficial effect of immunotherapy on airway symptoms and the allergen-specific Th2 response. T cell triggering below a certain threshold will not affect the effector function of the Th2 cell response, and this form of immunotherapy will not influence the airway symptoms. A low to intermediate level of T cell activation will induce and/or promote the Th2 response, resulting in deterioration of

AHR and eosinophilia, whereas immunotherapy strategies inducing strong systemic T cell activation will abrogate the Th2 response and ameliorate airway symptoms.

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