

Inhalation Tolerance Is Induced Selectively in Thoracic Lymph Nodes but Executed Pervasively at Distant Mucosal and Nonmucosal Tissues¹

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Under immunogenic conditions, both the site of initial Ag exposure and consequent T cell priming in specific draining lymph nodes (LNs) imprint the ensuing immune response with lasting tissue-selective tropism. With respect to immune tolerance, whether the site of tolerance induction leads to compartmentalized or, alternatively, pervasive tolerance has not been formally investigated. Using a murine model of inhalation tolerance, we investigated whether the induction of respiratory mucosal tolerance precludes the development of de novo Th2 sensitization upon subsequent exposure to the same Ag at distant mucosal (gut) and nonmucosal (cutaneous) sites. By tracking the proliferation of CFSE-labeled OVA-TCR transgenic CD4⁺ T cells upon OVA inhalation in vivo, we defined the site of tolerance induction to be restricted to the thoracic LNs. Expectedly, inhalation tolerance prevented de novo Th2 sensitization upon subsequent exposure to the same Ag at the same site. Importantly, although gut- and skin-draining LNs were not used during tolerance induction, de novo Ag-specific proliferation and Th2 differentiation in these LNs, as well as memory/effector Th2 responses in the gut (allergic diarrhea) and skin (late-phase cutaneous responses) were inhibited upon immunogenic challenge to the same Ag. Interestingly, this pervasive tolerogenic phenotype was not associated with the presence of suppressive activity throughout the lymphatics; indeed, potent suppressive activity was detected solely in the spleen. These data indicate that while inhalation tolerance is selectively induced in local thoracic LNs, its tolerogenic activity resides systemically and leads to pervasive immune tolerance in distant mucosal and nonmucosal sites. *The Journal of Immunology*, 2006, 176: 2568–2580.

The skin and mucosae amount to a vast surface area under continuous exposure to foreign Ags. The immune system is thus faced with the daunting task of discriminating between pathogenic and nonpathogenic Ags to generate appropriate immune responses. In the majority of individuals, innocuous environmental allergens are tolerated by the immune system inasmuch as they fail to provoke deleterious immune responses. However, in a growing fraction of the industrialized world, contact, ingestion, or inhalation of such allergens provokes harmful and sometimes fatal allergic responses (1, 2). It appears that the nature of the Ag, the specific tissue microenvironment at the site of initial encounter, and molecular events in the draining lymph node (LN)³

are critical in establishing whether long-lived immunity or tolerance prevails (3–8).

Immune tolerance has been shown to arise via several mechanisms, including the induction of anergy (9–11), clonal deletion of Ag-specific lymphocytes (12), or active immune regulation mediated by regulatory cells secreting TGF- β (13–17), IFN- γ (18–20), or IL-10 (21, 22). The dysregulation of these tolerogenic processes plays an important role in the emergence of aberrant immune responses to harmless environmental or self Ags (23, 24). Although the molecular basis underlying these mechanisms remains an area of intense investigation, comparatively little is known with respect to the extent by which immune tolerance is secured in diverse secondary lymphoid organs. This is pertinent not only to understand how tolerance is averted in autoimmune disease and allergy, but also to our understanding of whether tolerance induced at one site can preclude subsequent allergic sensitization to the same Ag anywhere in the body.

Current evidence suggests that upon stimulation in local draining LNs, naive T cells are imprinted with a unique repertoire of chemoattractant receptors (25–29) and adhesion molecules (30–32) that privilege them to survey tissues most likely to contain their cognate Ag. Indeed, recent observations have established that the local LN microenvironment and the tissue origin of Ag-bearing dendritic cells are major determinants in the programming for tissue-selective homing properties (33–36). Whereas it makes teleological sense that immune-effector responses are compartmentalized, it may be to our advantage that, regardless of the site of entry, the whole body is protected from developing aberrant responses to innocuous Ags.

The objective of this study was to investigate whether the induction of tolerance at one site precludes the development of subsequent allergic sensitization to the same Ag at a distant LN and

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³ Abbreviations used in this paper: LN, lymph node; Ad, adenoviral; BAL, bronchoalveolar lavage; GG, gene gun; GI, gastrointestinal; i.d., intradermal; i.n., intranasal; MNC, mononuclear cell; PAS, periodic acid-Schiff; TARC, thymus- and activation-regulated chemokine.

tissue compartment. We show that the induction of tolerance via the respiratory mucosa leads to a tolerogenic response initially confined to local thoracic LNs, but whose activity resides systemically, preventing de novo Ag-specific proliferation and Th2 differentiation, as well as memory/effector Th2 responses, in distant gut- and skin-draining LNs and tissues, respectively.

Materials and Methods

Animals

Female BALB/c and DO11.10 TCR transgenic mice (6–8 wk old) were purchased from Charles River Laboratories and housed in specific pathogen-free conditions following a 12-h light-dark cycle. DO11.10 TCR transgenic mice were bred in the central animal facility at McMaster University. Experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Induction of inhalation tolerance

Mice were subjected to a well-characterized protocol of inhalation tolerance (37) involving daily 20-min exposures to aerosolized OVA (grade V protein, 1% w/v in 0.9% saline; Sigma-Aldrich) for 10 consecutive days. The OVA aerosol was generated by a Bennet/Twin nebulizer operating at an airflow rate of 10 L/min. To determine the site of *in vivo* T cell proliferation in secondary lymphoid organs, mice were sacrificed at several time points throughout the tolerance protocol. To investigate the consequence of inhalation tolerance on de novo Th2 sensitization at distant mucosal and nonmucosal sites, mice were rested for a period of ~4 wk post-tolerance induction before undergoing respiratory mucosal, *i.e.*, or cutaneous Th2 sensitization protocols to the same Ag, as detailed below.

Th2 sensitization protocols

Tolerized or nontolerized mice were subjected to previously published protocols of Th2 sensitization via the respiratory mucosa, parenteral/*i.p.*, or cutaneous routes. For respiratory mucosal sensitization, mice were exposed to aerosolized OVA in the presence of exogenous GM-CSF expressed via adenoviral (Ad) gene transfer to the airway (38). We have shown previously only concurrent exposure to OVA and Ad/GM-CSF, but not a control Ad vector, nor Ad/GM-CSF alone, is able to elicit allergic sensitization and eosinophilic inflammation (38). Briefly, a replication-deficient human type 5 Ad vector encoding murine GM-CSF (Ad/GM-CSF) (39) was delivered intranasally (*i.n.*) to isoflurane-anesthetized mice at a dose of 3×10^7 PFU 1 day before OVA aerosolization. Sensitization via the parenteral route was achieved by *i.p.* injections of OVA, as previously described (40). Briefly, mice were sensitized twice, 5 days apart, by *i.p.* injection of 8 μ g of OVA (Sigma-Aldrich) and 4 mg of aluminum hydroxide (Sigma-Aldrich) in a total volume of 0.5 ml of sterile PBS. Sensitization to OVA via the skin was achieved by way of particle bombardment of plasmid OVA-coated gold particles via the Helios gene gun (GG) (Bio-Rad) (41). We have reported previously that delivery of plasmids expressing OVA, but not luciferase cDNA or empty control plasmids, nor delivery of gold particles alone, leads to OVA-specific Th2 immunity (41). Briefly, each animal received three nonoverlapping deliveries of gold particles coated with OVA-expressing plasmids, totaling 1 μ g of plasmid per inoculation (*i.e.*, 0.33 μ g of DNA per delivery), onto the ventral abdominal skin on three occasions at weekly intervals. The plasmid used for these studies was a modified version of pcDNA3.1⁺ (Invitrogen Life Technologies) containing the cDNA for OVA under control of the human CMV intermediate-early promoter. Preparation of DNA-coated gold particles was performed, as previously described (41).

CD4⁺CD62L⁺ T cell isolation, CFSE staining, and adoptive transfer

Naive CD4⁺ T cells from DO11.10 TCR transgenic mice were isolated from pooled LNs (inguinal, axillary, cervical, thoracic, brachial, popliteal, and mesenteric) using the murine CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec). Briefly, LNs were triturated between the ends of sterile frosted slides, filtered through 40- μ m nylon cell strainers (BD Falcon), washed at 1200 rpm for 10 min at 4°C, and then resuspended in PBS (0.5% BSA and 2 mM EDTA). Single-cell LN suspensions were enriched for CD4⁺ T cells by first depleting B220⁺, CD8a⁺, CD11b⁺, CD49b⁺, and Ter-119⁺ cells by indirect magnetic bead labeling, then positively selected for CD62L⁺ cells using the autoMACS cell sorter (Miltenyi Biotec), according to manufacturer specifications. The purity (percentage of CD4⁺CD62L⁺ KJ1-26⁺) of the population was typically greater than 95%. Next, the donor CD4⁺CD62L⁺ T cells were resuspended in PBS/5% FBS,

stained with 5 μ M CFSE at room temperature for 5 min, and then washed three times in PBS/5% FBS at 1200 rpm for 5 min at 20°C. The donor transgenic T cells were then adoptively transferred by tail vein injection into naive syngeneic BALB/c mice 24 h before OVA exposure (5×10^6 cells in 200 μ l of sterile PBS *i.v.*).

In vivo proliferation

Following adoptive transfer of CFSE^{bright} OVA-TCR transgenic T cells into syngeneic BALB/c recipients, mice were exposed to aerosolized OVA under tolerizing or priming conditions (*i.e.*, a GM-CSF-enriched airway microenvironment). At the indicated time points, LN cells were harvested, washed twice in FACS buffer (0.5% BSA in PBS), and then stained. To minimize nonspecific binding, cells were preincubated with FcBlock (BD Pharmingen). Donor transgenic T cells were visualized in recipient LNs by flow cytometry using the clonotypic TCR Ab KJ1-26:PE, anti-mouse CD3:allophycocyanin, and anti-mouse CD4:PerCP (all purchased from BD Pharmingen). Diminution of CFSE intensity on gated donor transgenic T cells served as an indicator of Ag-specific proliferation *in vivo*. For each Ab combination, 2×10^6 cells were incubated with Abs at 4°C for 30 min, then washed and treated with second-stage reagents. All data were collected on a LSRII flow cytometer (BD Biosciences) and were analyzed using WinMDI software (The Scripps Research Institute).

Splenocyte and LN cell culture

Spleens or LNs (axillary, inguinal, deep cervical, supercervical, thoracic, mesenteric, or Peyer's patches) were excised at various time points during tolerance induction, or following *i.p.*, cutaneous, or respiratory mucosal Th2 sensitization protocols, and placed into sterile tubes containing sterile HBSS on ice. Spleens/LNs were triturated between the ends of sterile frosted slides, and the resulting cell suspension was filtered through 40- μ m nylon cell strainers (BD Falcon), then washed at 1200 rpm for 10 min at 4°C. RBCs were lysed from spleen suspensions by adding 1 ml of ammonium-chloride-potassium lysis buffer (0.5 M NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA (pH 7.2–7.4)) for 1 min. For culturing, splenocytes and dispersed LN cells were washed with HBSS and then resuspended in RPMI 1640 medium supplemented with 10% FBS (Sigma-Aldrich), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen Life Technologies), and 0.1% 2-ME (Invitrogen Life Technologies). Cells were cultured in medium alone or with 40 μ g of OVA/well and seeded at 8×10^5 cells/well (spleen) or 5×10^5 cells/well (LN) in a flat-bottom, 96-well plate (BD Biosciences). Following 120 h of culture incubation, supernatants were harvested and stored at –20°C for cytokine detection.

In vitro LN cell proliferation

Ag-specific proliferation was determined by a [³H]thymidine incorporation *in vitro* assay. Briefly, 1 μ Ci/well [³H]thymidine (PerkinElmer Life Sciences) was added to the last 18 h of a 3-day culture, in the presence or absence of OVA (40 μ g/well), and proliferative responses were measured by cell uptake of [³H]thymidine. Cells were harvested using a Filtermate harvester (Packard BioScience), quantified using TopCount NXT microplate scintillation and luminescence counter (Packard BioScience), and expressed as the mean cpm $\pm$ SD of triplicate wells, where applicable.

In vitro DO11.10 T cell proliferation assay

To assess the suppressive activity within various secondary lymphoid organs in mice actively undergoing respiratory mucosal tolerance, whole LN/spleen cultures (seeded at 5×10^5 cells/well) were individually prepared from thoracic LNs, cervical LNs, inguinal/axillary LNs, Peyer's patches, and spleens from actively tolerized (seven OVA exposures) or naive mice (pooled from five mice). Increasing concentrations (0, 1×10^4 , 5×10^4 , 2×10^5 , and 5×10^5) of naive CD4⁺CD62L⁺ DO11.10 OVA-TCR transgenic T cells were cocultured with LN or spleen cells. Cultures were stimulated *in vitro* with OVA or medium for 72 h. Proliferation of DO11.10 T cells was assessed by a [³H]thymidine incorporation *in vitro* assay, as outlined above.

Oral Ag-induced diarrhea

Two weeks following *i.p.* Th2 sensitization, mice were subjected to a recently characterized protocol of Ag-induced allergic diarrhea (42). Briefly, mice were held in the supine position and orally administered 50 mg of OVA protein in 250 μ l of sterile PBS by gavage every other day up to seven times. Before each intragastric challenge, mice were deprived of food for 4 h to limit the extent of Ag degradation in the stomach. Clinical scoring of diarrhea was assessed by visually monitoring mice for up to 1 h following challenge, by multiple observers. Mice demonstrating profuse liquid stool were scored as positive.

Late-phase cutaneous responses

Late-phase cutaneous responses were induced in mice by intradermal (i.d.) injection with 10 μ g of OVA in 10 μ l of sterile PBS into one ear, and vehicle (sterile PBS) into the opposite ear, 1 wk postcutaneous Th2 sensitization (41). Ear thickness was measured before and at several time points after injection using a modified low-tension thickness gauge (Dyer). Mice were sacrificed 48 h later, and ears were fixed in 10% Formalin, and then sectioned and stained with H&E. The total number of eosinophils was enumerated in each section, in a blinded manner, under $\times 400$ magnification. Data are expressed as the number of eosinophils per mm² ear tissue as determined by Northern Eclipse software (Empix Imaging).

Serum collection and bronchoalveolar lavage (BAL)

Peripheral blood was collected by retro-orbital bleeding. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C, and stored as aliquots at -20°C . BAL was performed, as previously described (43). Briefly, the lungs were dissected and the trachea was cannulated with a polyethylene tube (BD Biosciences). The lungs were lavaged twice with PBS (0.25 ml, followed by 0.2 ml); ~ 0.3 ml of the instilled fluid was consistently recovered. Total cell counts were determined in a blinded manner using a hemocytometer. Each BAL sample was centrifuged, and the supernatant was stored at -20°C for cytokine and chemokine detection. The cell pellet was resuspended in PBS, and smears were prepared by cytocentrifugation (Thermo Shandon) at 300 rpm for 2 min. BAL smears were stained with the Protocol Hema 3 stain set (Fisher Scientific). Differential cell counts of BAL smears were determined in a blinded manner from at least 500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells (MNCs). Where applicable, lung tissue was fixed in 10% Formalin and embedded in paraffin. Sections, 3 μ m thick, were stained with H&E (for visualization of leukocytes and histopathological features) or periodic acid-Schiff (PAS) (for detection of goblet cells).

Cytokine and Ig measurement

Cytokine/Chemokine content was determined using ELISA kits purchased from R&D Systems for murine IL-4, IL-5, IL-10, IL-13, IFN- γ , thymus- and activation-regulated chemokine (TARC), MCP-1, and RANTES. Each of these assays has a threshold of detection between 1.5 and 5 pg/ml. Levels of OVA-specific serum IgE and IgG1 were measured, as previously described (37, 43). Units of OVA-specific Igs were determined relative to in-house standardized serum, obtained from mice sensitized to OVA through conventional i.p. sensitization (43). Sample Ig levels are expressed in U/ml relative to standard mouse sera.

Data analysis

Data are expressed as mean $\pm$ SEM, unless otherwise noted. Statistical analysis was performed using SigmaStat software (SPSS). Results were interpreted using ANOVA, followed by Fisher's least significant difference post hoc test analysis or Student's *t* test, where appropriate. A value of *p* < 0.05 was considered statistically significant.

Results

Inhaled Ag leads to robust proliferative responses in thoracic LNs in vivo

The precise site(s) of T cell proliferation during tolerance induction to harmless airborne Ags is not well defined. To assess which secondary lymphoid organ(s) takes part in immune tolerance induction via the respiratory mucosa, we used a transgenic TCR adoptive transfer system to identify the site(s) in which Ag-specific T cells proliferated in vivo. To this end, 5×10^6 CFSE^{bright} DO11.10 KJ1-26⁺ OVA-TCR transgenic CD4⁺ T cells (donor transgenic T cells) were adoptively transferred to naive syngeneic BALB/c recipients 1 day before commencement of daily exposures to aerosolized OVA for 3 or 10 consecutive days (Fig. 1A). Following OVA aerosolization, we examined distinct secondary lymphoid organs using diminution of CFSE intensity on gated donor transgenic T cells as an indicator of Ag-specific proliferation. Specifically, we dissected LNs from the head and neck region (super and deep cervical), the airways (thoracic), the gastrointestinal (GI) tract (mesenteric and Peyer's patches), and peripheral cutaneous sites (inguinal and axillary).

In mice left unexposed to Ag (naive recipients), the transferred donor transgenic T cells did not undergo Ag-specific proliferation in vivo in any LN examined; the majority of donor transgenic T cells (97–99%) remained in the CFSE^{bright} undivided peak (Fig. 1, B and D). In naive recipient LNs, donor transgenic T cells were normally detected at a frequency of $\sim 0.35\%$ (i.e., ~ 1 in 300 gated MNC LN cells), with notable exception in the Peyer's patches, which consistently contained $< 0.1\%$ (Fig. 1C). However, following OVA aerosolization (tolerizing conditions), we consistently observed robust Ag-specific proliferation of the donor transgenic T cell pool primarily in the thoracic LNs (Fig. 1, B and F). The degree of proliferation correlated with the number of exposures. From 3 to 10 OVA exposures, we observed an increase in the proportion (Fig. 1, B and D) and absolute number of total (Fig. 1E) and proliferating (Fig. 1F) donor transgenic T cells, which was accompanied by a decrease in the undivided fraction (from $32.3 \pm 5.4\%$ to $8.5 \pm 1.5\%$). This translated to an expansion of the donor transgenic T cell pool preferentially within the thoracic LNs from 0.35% at baseline to $\sim 1\%$ following 3 and to $\sim 1.5\%$ after 10 OVA exposures (Fig. 1C). To a lesser extent, we also observed Ag-specific proliferation in cervical LNs, but only after 10 OVA exposures, with $\sim 50\%$ of donor transgenic T cells having undergone several rounds of Ag-specific proliferation (Fig. 1, B and D). Despite this, the overall percentage of donor transgenic T cells in cervical LNs decreased compared with baseline (Fig. 1C). In fact, we consistently observed a continual reduction in the number of donor transgenic T cells in all distal LNs (except thoracic) throughout the tolerance protocol (Fig. 1C). Of note, the absolute number of proliferating donor transgenic T cells in cervical LNs was less than one-tenth the number in the thoracic LNs (Fig. 1F). Importantly, within distal mucosal (mesenteric LNs) and nonmucosal LNs (inguinal/axillary LNs), we observed minimal T cell proliferation during tolerance induction (Fig. 1, B and F). Although we did detect a statistically significant decrease in the undivided fraction of donor transgenic T cells in mesenteric LNs and inguinal/axillary LNs from 3 to 10 OVA exposures (Fig. 1D), this corresponded, proportionally, to very few dividing donor transgenic T cells (0.015–0.02% of gated MNC LN cells; Fig. 1C) and also very few in absolute number (Fig. 1F). A similar finding was evident in the Peyer's patches. In agreement with this, we did not observe an enlargement of mesenteric LNs and inguinal/axillary LNs upon dissection (data not shown), as was evident for the thoracic LN (and to a lesser degree the cervical LNs), and as would be expected to accompany an active immune response (see Fig. 2A). We also examined the expression of CD69 on donor transgenic T cells in LNs following 2 and 3 OVA exposures. Consistent with the proliferation data, we observed ~ 2 -fold increase in the proportion of donor transgenic T cells expressing CD69 in thoracic compared with distal LNs (inguinal/axillary, mesenteric, or Peyer's patches; data not shown).

Next, we compared the in vivo proliferative response to OVA inhalation under tolerizing conditions with that evoked in a GM-CSF-enriched airway microenvironment (priming conditions). As shown in Fig. 2A, exposure to OVA under priming conditions led to a marked cellular expansion of cervical and thoracic LNs, including a greater expansion of donor transgenic T cells, compared with OVA exposure alone. Indeed, a far greater fraction of the donor transgenic T cell pool had undergone several rounds of proliferation in both cervical and thoracic LNs in primed vs tolerized mice (Fig. 2C). By 10 exposures, $\sim 97\%$ of the resident pool of donor transgenic T cells in thoracic LNs (and also cervical LNs) had divided in primed animals (Fig. 2, B and C). Importantly, these contrasting Ag exposure protocols ultimately led to distinct physiological outcomes. In fact, histological analysis of lung tissue

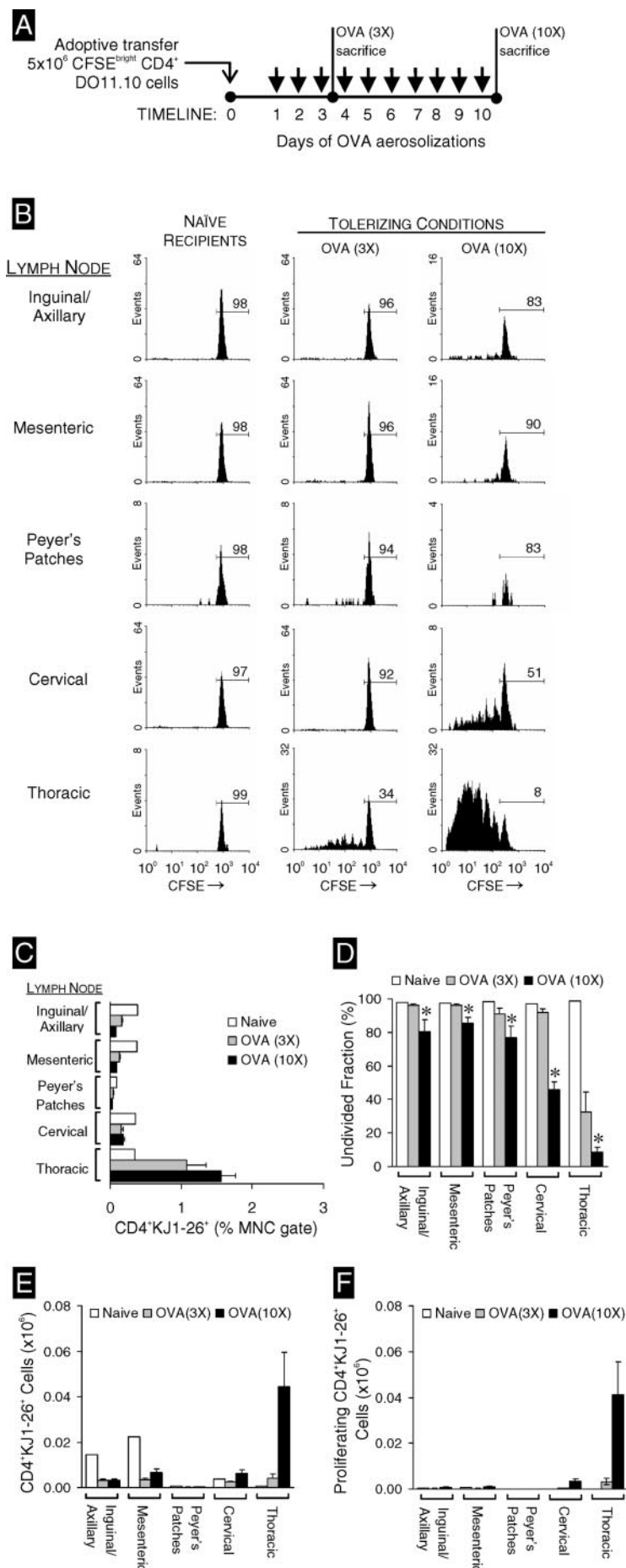


FIGURE 1. Ag inhalation under tolerizing conditions leads to Ag-specific proliferation of CD4⁺ DO11.10 donor transgenic T cells predominantly in local thoracic and cervical, but not distant mucosal and nonmucosal, LNs in vivo. **A**, Mice were injected i.v. with 5×10^6 CFSE-labeled DO11.10 KJ1-26⁺ OVA-TCR transgenic CD4⁺ T cells 24 h before commencement of daily exposures to aerosolized OVA for 3 or 10 consecutive days. **B**, Following 3 or 10 OVA exposures (or in naive recipients), mice were sacrificed, and the CFSE intensity on gated CD4⁺KJ1-26⁺ donor transgenic T cells was determined in local (cervical and thoracic) and distant (inguinal/axillary, mesenteric, and Peyer's patches) LNs by flow cytometry. The percentage of undivided donor transgenic T cells is marked on each histogram. The percentage of expansion (**C**) and fraction of undivided (**D**), CD4⁺KJ1-26⁺ donor transgenic T cells within local and distant LNs in naive recipients or following OVA exposure. Absolute number of total (**E**) and proliferating (**F**) CD4⁺KJ1-26⁺ donor transgenic T cells in LNs of naive or tolerized recipients. *, $p < 0.05$ between 3 and 10 OVA exposures by Student's *t* test. Results are representative of three independent experiments with two to three mice per group.

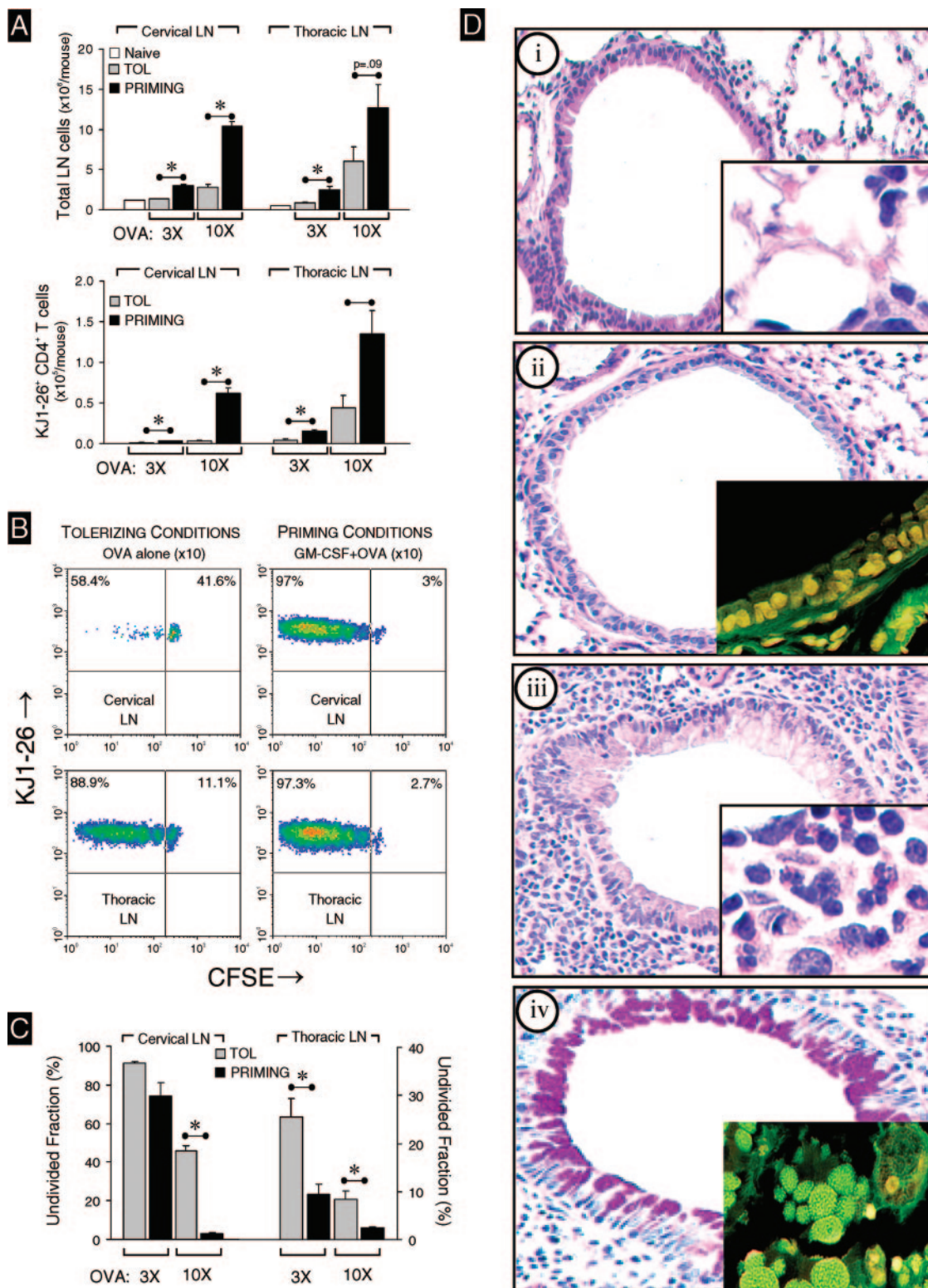


FIGURE 2. Mitigated Ag-specific T cell responses and local tissue inflammation following Ag inhalation under tolerizing vs priming conditions. Mice were injected i.v. with 5×10^6 CFSE-labeled DO11.10 KJ1-26⁺ OVA-TCR transgenic CD4⁺ T cells 24 h before commencement of daily exposures to aerosolized OVA for 3 or 10 consecutive days in the presence or absence of concurrent adjuvant delivery. **A**, Following 3 or 10 OVA exposures in tolerizing or priming conditions (or in naive recipients), mice were sacrificed, and the total cellular expansion and absolute number of CD4⁺KJ1-26⁺ donor transgenic T cells were determined in local cervical and thoracic LNs. **B**, Typical proliferative expansion of CD4⁺KJ1-26⁺ donor transgenic T cells following 10 OVA exposures under tolerizing vs priming conditions in local cervical and thoracic LNs. Quadrant statistic delineates fraction of divided-to-undivided populations. **C**, Overall fraction of undivided CD4⁺KJ1-26⁺ donor transgenic T cells following 3 or 10 OVA exposures under tolerizing or priming conditions. *, $p < 0.05$ tolerizing vs priming conditions by Student's *t* test. **D**, Representative light photomicrographs of paraffin-embedded sections of murine lung tissue 24 h following 10 OVA exposures under tolerizing (*i* and *ii*) or priming (*iii* and *iv*) conditions. *i* and *iii*, Stained with H&E; *ii* and *iv*, stained with PAS. Insets in *ii* and *iv*, Depict PAS-stained sections viewed under color inversion to better define goblet cell hyperplasia. Original magnification of panels, $\times 200$. Magnification of insets, $\times 400$.

from mice exposed to OVA under priming conditions revealed extensive peribronchial and perivascular eosinophilic inflammation, with marked goblet cell hyperplasia and mucus production, unlike mice exposed to OVA under tolerizing conditions that did not present with any overt immune pathology (Fig. 2D). This was also associated with a near 5-fold increase in total cells (data not shown) and significantly higher eosinophils ($26 \pm 6.7\%$ vs $0.2 \pm 0.2\%$) in BAL fluid of primed vs tolerized mice. Taken together, these data demonstrate that, under tolerizing conditions, inhalation of harmless airborne Ags leads to an active immune response characterized by a comparatively mitigated T cell expansion in local secondary lymphoid organs. This ultimately leads toward a state of Ag unresponsiveness in the target organ (i.e., lung) and deviation from an otherwise detrimental Th2 immune phenotype. Importantly, these data point to the local lung-draining thoracic LNs as the predominant site for the induction of T cell tolerance *in vivo*.

Inhalation tolerance prevents *de novo* respiratory mucosal Th2 sensitization

To address the consequence of respiratory mucosal tolerance on subsequent Ag exposure at distant sites, we first ascertained that

tolerance induction via the respiratory mucosa was able to maintain a lasting state of Ag unresponsiveness in the same tissue compartment (i.e., airways). Thus, mice were initially exposed to aerosolized OVA (tolerized) or left unexposed to Ag (nontolerized), rested for a 4-wk period, and then re-exposed to OVA by subjecting them to a protocol of respiratory mucosal Th2 sensitization (Fig. 3A). To this end, we initially performed a comprehensive analysis of local draining LNs (thoracic, supracervical, and deep cervical) at various time points throughout sensitization and monitored for the evolution of a Th2 immune response. Indeed, we observed that prior Ag exposure under tolerizing conditions prevented Ag-specific proliferation and Th2 differentiation (IL-4, IL-5, and IL-13 production) within local draining LNs, unlike nontolerized mice, which displayed clear evidence of developing Th2 immunity (Fig. 3, B and C). Moreover, a similar inhibition of Ag-specific proliferation and Th2 cytokine production was also observed in cultured splenocytes of tolerized mice compared with nontolerized controls (data not shown). Consistent with the above data, only the nontolerized group developed robust allergic airways inflammation, with a significant 2.5-fold increase in total

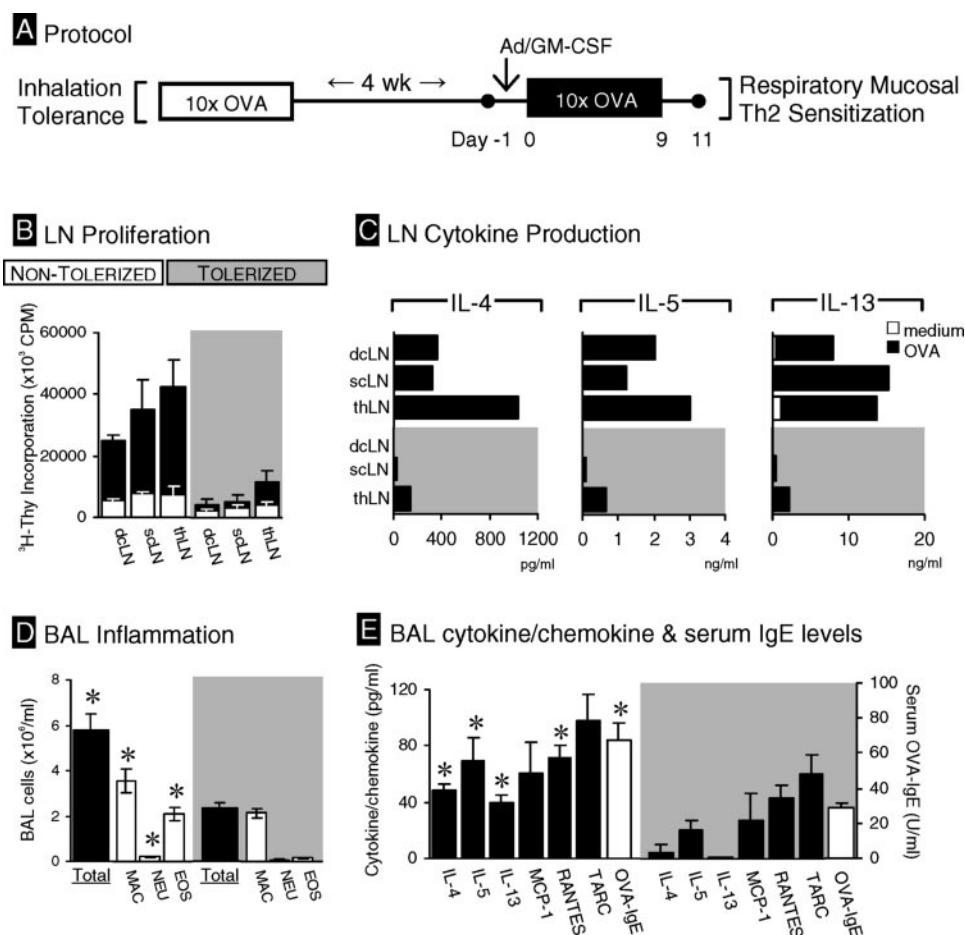


FIGURE 3. Tolerance induction via the respiratory mucosa prevents subsequent *de novo* Th2 differentiation in local draining LNs and Th2 effector responses in the airway, following a protocol of respiratory mucosal Th2 sensitization. **A**, Mice were initially exposed to aerosolized OVA daily for 20 min over a period of 10 consecutive days (tolerized, shaded) or left unexposed to Ag (nontolerized), and then rested for a 4-wk period before subjecting them to a protocol of respiratory mucosal Th2 sensitization to the same Ag (OVA). Mice were sacrificed during sensitization (day 7) for BAL cytokine/chemokine measurements or 48 h postsensitization (day 11) for *in vitro* LN culture, serum IgE levels, and the assessment of airway inflammatory outcomes. **B**, Ag-specific proliferation of OVA-stimulated (thoracic (th), supracervical (sc), and deep cervical (dc)) LN cells *in vitro* (LNs individually pooled from three to four mice; mean $\pm$ SD of triplicate wells). **C**, Th2 cytokine (IL-4, IL-5, and IL-13) production from OVA-stimulated LN cells *in vitro* (LNs individually pooled from three to four mice). **D**, Total (■) and differential (□) cellular analysis in BAL fluid (mean $\pm$ SEM; $n = 4$ –5 mice/group; *, $p < 0.05$, nontolerized vs tolerized). **E**, Cytokine/Chemokine levels in the BAL fluid (■) and OVA-specific serum IgE (□), as measured by ELISA (mean $\pm$ SEM; $n = 4$ –5 mice/group; *, $p < 0.05$, nontolerized vs tolerized). BAL IL-13 and TARC levels expressed as $\times 10^1$ pg/ml. Data are representative of two independent experiments.

BAL cells compared with tolerized mice, including a striking 15-fold increase in BAL eosinophils (Fig. 3D). In addition, we observed a significant increase in Th2-associated cytokines (IL-4, IL-5, and IL-13) along with higher levels of proinflammatory chemokines (MCP-1, RANTES, and TARC) in the BAL fluid of nontolerized vs tolerized mice (Fig. 3E). Of note, we did not observe increased levels of the immunoregulatory cytokine IL-10 in the BAL fluid of tolerized mice (data not shown). Furthermore, the *de novo* induction of serum OVA-specific IgE following respiratory mucosal Th2 sensitization was significantly suppressed by 60% in mice previously tolerized to OVA (Fig. 3E). Altogether, these data demonstrate that prior respiratory exposure to harmless airborne Ags under tolerizing conditions prevents *de novo* Th2 differentiation and immune inflammatory responses to the same Ag at the same site.

Inhalation tolerance prevents Ag-specific proliferation and Th2 differentiation in distant mucosal and nonmucosal LNs

That the induction of the tolerogenic response developed almost exclusively in local lung-draining LNs during Ag inhalation while sparing gut- and peripheral skin-draining LNs prompted us to investigate whether inhalation tolerance was as equally likely to halt sensitization to the same Ag at a distant LN microenvironment. Therefore, mice were initially tolerized to OVA via the respiratory tract, rested for a 4-wk period, and then subjected to distinct protocols of Th2 sensitization initiated at i.p. or cutaneous sites (Fig. 4A). Sensitization to OVA via i.p. injections in nontolerized mice elicited robust Ag-specific proliferation in distant mucosal gut-associated mesenteric LNs, and to a lesser extent in Peyer's patches (Fig. 4B). Conversely, mice previously tolerized to OVA via the respiratory mucosa failed to mount Ag-specific proliferative responses in distant mucosal gut-associated LNs and Peyer's patches. Of particular significance, the primary lymphatic drainage sites following Ag delivery into peritoneal cavity are the LNs of the mediastinum (thoracic) before systemic dissemination (44). Thus, the i.p. route may not be ideally suited to address whether tolerance induction via the lung-draining LNs can have an impact on *de novo* Th2 differentiation in distant, unrelated LNs. Incidentally, and consistent with our previous observations (Fig. 3B) (37), the proliferative responses normally seen in thoracic LNs post-i.p. sensitization were also ablated in tolerized mice (Fig. 4B).

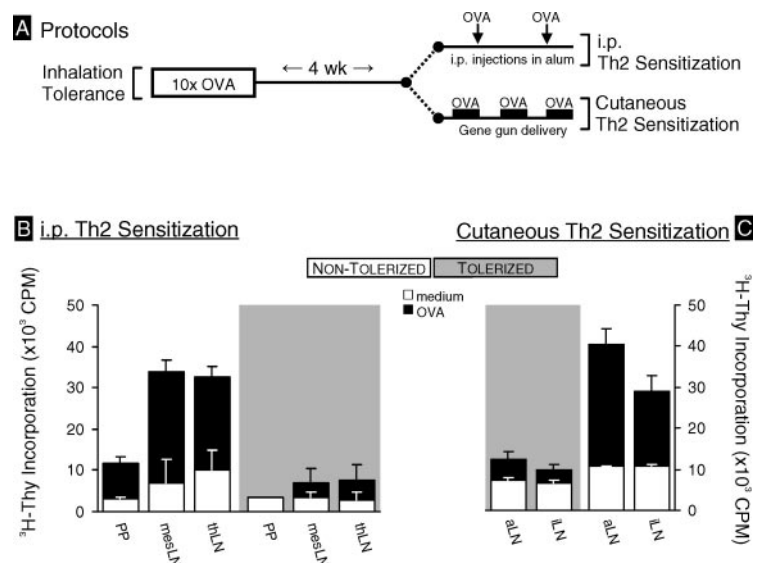
To directly test whether Ag inhalation under tolerizing conditions leads to pervasive immune tolerance at distant LNs, a system

was required whereby sensitization was solely confined to LNs distant to the principal nodes involved in inhalation tolerance. Recently, we have shown that cutaneous Ag sensitization via GG particle bombardment with gold-coated OVA-encoding plasmids resulted in the generation of Ag-specific Th2 immunity preferentially in skin-draining, but nonthoracic-draining, LNs (41). In agreement with our previous findings, cutaneous sensitization to OVA elicited robust Ag-specific proliferation in local skin-draining inguinal and axillary LNs in nontolerized mice (Fig. 4C). However, mice previously tolerized to OVA via the respiratory mucosa were unable to mount Ag-specific proliferative responses in distant nonmucosal skin-draining LNs following cutaneous sensitization. In addition, we examined the production of a number of Th2-affiliated cytokines upon Ag stimulation of harvested LN cells and Peyer's patches *in vitro* as an indication of Th2 differentiation. In nontolerized mice, i.p. and cutaneous Ag sensitization induced clear signs of Th2 differentiation, as evidenced by the robust production of IL-4, IL-5, and IL-13 from either gut-associated mesenteric LN and Peyer's patch cells or skin-draining (inguinal and axillary) LN cells, respectively (Fig. 5). However, tolerized mice were markedly prevented from undergoing *de novo* Th2 differentiation upon subsequent OVA exposure, even in distant mucosal and nonmucosal LNs and, importantly, even in LNs distant to the original site of tolerance instruction. This inhibition of Th2 differentiation in mucosal and nonmucosal LNs was not due to the increased production of Th2-opposing cytokines (i.e., Th1 associated), as we did not observe enhanced IFN- γ production by tolerized LN cells (data not shown).

*Inhalation tolerance prevents the *de novo* induction of Ag-specific IgE and systemic Th2 immune responses by i.p. or cutaneous routes*

Next, we investigated whether respiratory mucosal tolerance could prevent the induction of *de novo* humoral immune responses upon subsequent exposure to the same Ag introduced to distant mucosal and nonmucosal LNs. Similar to our previous findings (Fig. 3E) (37), respiratory mucosal tolerance significantly prevented the generation of OVA-specific serum IgE otherwise induced by i.p. Th2 sensitization protocols (Table I). In addition, the levels of OVA-specific serum IgE normally induced following cutaneous Ag sensitization were also markedly reduced in mice previously tolerized to OVA. Of interest, Ag re-exposure via either i.p. or cutaneous

FIGURE 4. Tolerance induction via the respiratory mucosa prevents subsequent *de novo* Ag-specific proliferation in distant mucosal and nonmucosal LNs. **A**, Mice were initially exposed to aerosolized OVA daily for 20 min over a period of 10 consecutive days (tolerized, shaded) or left unexposed to Ag (nontolerized), and then rested for a 4-wk period before subjecting them to a protocol of i.p. or cutaneous Th2 sensitization to the same Ag (OVA). **B**, Ag-specific proliferation of OVA-stimulated Peyer's patches (PP), mesenteric LNs (mesLNs), or thoracic LNs (thLNs) *in vitro* (LN/PPs individually pooled from four mice) harvested 72 h post-i.p. Th2 sensitization from mice initially tolerized or nontolerized. **C**, Ag-specific proliferation of OVA-stimulated LN cells *in vitro* (axillary (aLN) and inguinal (iLN); individually pooled from five mice) harvested 1 wk postcutaneous Th2 sensitization from mice initially tolerized or nontolerized. Data are expressed as mean $\pm$ SD of triplicate wells. Results are representative of at least two independent experiments.



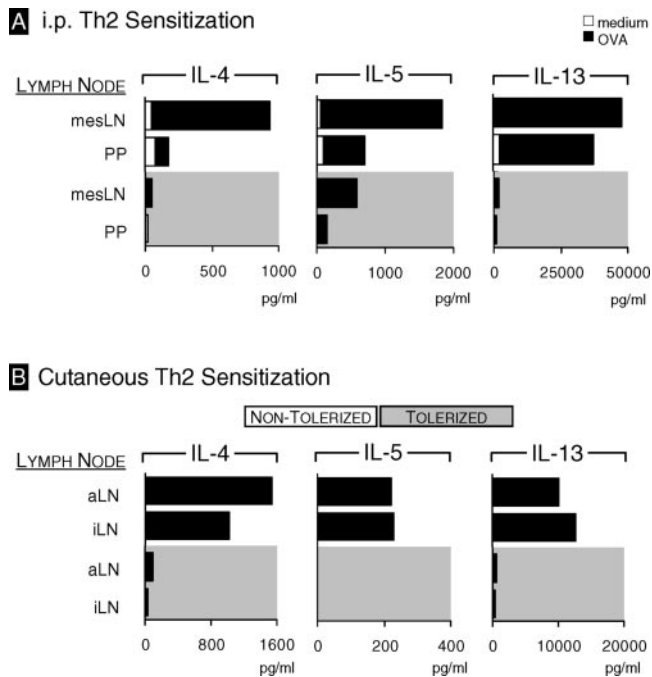


FIGURE 5. Tolerance induction via the respiratory mucosa prevents subsequent de novo Th2 differentiation in distant mucosal and nonmucosal LNs. Mice were initially exposed to aerosolized OVA daily for 20 min over a period of 10 consecutive days (tolerized, shaded) or left unexposed to Ag (nontolerized), and then rested for a 4-wk period before subjecting them to a protocol of i.p. or cutaneous Th2 sensitization to the same Ag (OVA). *A*, Ag-specific Th2 cytokine (IL-4, IL-5, and IL-13) production in vitro by mesenteric LN (mesLN) or Peyer's patch (PP) cells (LN/PPs individually pooled from three to four mice) harvested 72 h post-i.p. Th2 sensitization from mice initially tolerized or nontolerized. *B*, Ag-specific Th2 cytokine (IL-4, IL-5, and IL-13) production in vitro by OVA-stimulated LN cells (axillary (aLN) and inguinal (iLN); individually pooled from three to four mice) harvested 72 h postcutaneous Th2 sensitization from mice initially tolerized or nontolerized. Data are representative of at least three independent experiments.

sensitization protocols actually led to an increase in the levels of OVA-specific serum IgG1 in mice previously tolerized to OVA compared with nontolerized controls (Table I). To further characterize the effects of prior respiratory mucosal tolerance on systemic markers of Th2 immunity, we also examined the production of Th2 cytokines by cultured splenocytes in nontolerized vs tolerized mice. As described in Table I, the production of IL-4, IL-5, and IL-13 by OVA-stimulated splenocytes in vitro was inhibited in mice previously tolerized to OVA via the respiratory mucosa, regardless of the site of Ag re-exposure (i.e., skin or i.p. route). Importantly, Th2 cytokine production was not completely inhibited, because the levels of IL-4, IL-5, and IL-13 were still increased in tolerized mice compared with naive controls (Table I).

Respiratory mucosal tolerance prevents Th2 allergic diarrhea responses at the gut mucosa

We next determined the impact of respiratory mucosal tolerance on Th2 effector/memory responses upon Ag challenge at a distant mucosal site. To this end, mice were initially tolerized to OVA via the respiratory tract, rested for a 4-wk period, sensitized to the same Ag by the i.p. route, and then 2 wk later repeatedly challenged with OVA (by oral gavage) while being monitored for GI dysfunction (i.e., Ag-induced allergic diarrhea; Fig. 6A). Consistent with the inhibitory effect of respiratory mucosal tolerance on Ag-specific proliferation and Th2 differentiation in gut-associated

LNs and Peyer's patches, and on the induction of IgE following i.p. priming (Figs. 4B and 5A), tolerized mice were markedly prevented from developing Ag-induced allergic diarrhea, unlike the nontolerized control mice, which developed profuse symptoms (Fig. 6B).

Respiratory mucosal tolerance prevents Th2 late-phase cutaneous responses

Next, we investigated the impact of respiratory mucosal tolerance on the development of Th2 effector/memory responses upon Ag challenge at a distant nonmucosal site. To this end, mice were initially tolerized to OVA via the respiratory tract, rested for a 4-wk period, sensitized to the same Ag by cutaneous GG bombardment, and then 1 wk later injected i.d. with OVA or vehicle (PBS) into the left and right ears, respectively (Fig. 7A). As depicted in Fig. 7B, OVA, but not PBS, injection led to a marked and statistically significant acute- and late-phase cutaneous response in nontolerized mice (compared with tolerized and naive control mice) as assessed by increases in ear thickness over several time points starting from baseline (0 h). Histological examination of ear tissue 48 h following i.d. OVA, but not PBS (data not shown), injection showed a pronounced influx of MNCs and eosinophils, particularly in the nontolerized group (Fig. 7C). Indeed, enumeration of ear tissue eosinophils was in agreement with the histological findings, demonstrating a marked increase in infiltrating eosinophils in ears recalled with OVA compared with tolerized and naive control mice (Fig. 7D). Of particular importance, tolerized mice also mounted a statistically significant acute-phase response following OVA ear challenge compared with baseline (Fig. 7B). This increase in ear thickness subsided rather slowly, and by 48 h remained inflamed compared with PBS-injected ears or naive controls (although statistically less inflamed than the nontolerized group). Upon further histological examination, we observed a statistically significant accumulation of eosinophils in the ears of nontolerized mice injected i.d. with OVA, compared with naive control mice or PBS-injected ears. Moreover, Th2 cytokine production (IL-4, IL-5, and IL-13) by OVA-stimulated ear-draining auricle LN cells in vitro was also suppressed in tolerized vs nontolerized mice following i.d. OVA challenge (data not shown).

Suppressive activity resides in the systemic compartment following inhalation tolerance

That the induction of respiratory mucosal tolerance was restricted to local lung-draining LNs in vivo raised the question of how tolerized mice are able to prevent de novo Th2 sensitization upon recall to the same Ag at distant mucosal and nonmucosal LNs. Thus, we harvested LN and spleen cells from actively tolerized (seven OVA exposures) or naive mice and determined which secondary lymphoid organs harbored suppressive activity. To this end, we examined the proliferation of naive CD4⁺ DO11.10 responder T cells cocultured with different LN or spleen cells from tolerized or naive mice in vitro (Fig. 8). Surprisingly, cells harvested from local lung-draining thoracic LNs were unable to suppress the proliferative expansion of DO11.10 responder T cells, despite the fact they were primarily involved during tolerance induction. In fact, the DO11.10 responder T cells actually proliferated greater when cocultured with thoracic LNs taken from tolerized mice compared with other LNs and with LNs harvested from naive mice. We did not observe suppressive activity in cervical LNs, mesenteric LNs (data not shown), or Peyer's patches. However, we did detect potent suppressive activity in the spleen compartment of tolerized mice, and to a lesser extent in nonmucosal skin-draining LNs.

Table I. OVA-specific serum Ig and systemic Th2-immune responses following parenteral/i.p. or cutaneous Th2 sensitization in previously tolerized mice^a

Sensitization Site	OVA-Specific Serum		Splenocyte In Vitro Recall ^c		
	IgE ^b	IgG1 ($\times 10^4$)	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (ng/ml)
Naïve controls	10 $\pm$ 6	<1	22.9 $\pm$ 1.7	<7	0.9 $\pm$ 0.3
Parenteral/i.p.					
Nontolerized	125 $\pm$ 38	1.1 $\pm$ 0.6	341 $\pm$ 109	4473 $\pm$ 1214	20.0 $\pm$ 1.1
Tolerized	31.0 $\pm$ 2.3*	42.3 $\pm$ 9.4*	113 $\pm$ 17*	1254 $\pm$ 299*	4.7 $\pm$ 0.9*
Cutaneous					
Nontolerized	160 $\pm$ 32	6.7 $\pm$ 1.5	234 $\pm$ 42	582 $\pm$ 76	8.8 $\pm$ 0.6
Tolerized	4.4 $\pm$ 3.0*	17.0 $\pm$ 1.4*	55 $\pm$ 11*	224 $\pm$ 131*	4.7 $\pm$ 1.2*

^a Nontolerized or tolerized mice were re-exposed to OVA via the skin or i.p. route under Th2-sensitizing conditions 4 wk posttolerance induction. Data are expressed as mean $\pm$ SEM; $n = 3-4$ /group. Results are representative of two independent experiments.

^b OVA-specific serum IgE and IgG1 was measured by ELISA and expressed as unit/milliliter.

^c Cytokine levels in culture supernatants.

* $p < 0.05$ compared with nontolerized mice.

Discussion

Although the nature of the Ag largely defines the type of immune response that is generated (tolerance or immunity), the site of initial Ag exposure conveys additional information. Current evidence suggests that during immunologic priming, the site and, in particular, the specific draining LN imprint the ensuing immune response with lasting tissue-selective tropism. This is, in part, accomplished by the acquisition of differential homing instructions, including, but not limited to, unique adhesion molecules (30–32) and chemoattractant receptors (25–29) that direct lymphocytes to tissues in which they are most likely to re-encounter their cognate Ag. Indeed, memory/effector T cells have been identified that selectively home to cutaneous (45–47) and various mucosal (30, 31, 33) tissues. Whether this tissue-selective paradigm is also evoked during tolerance induction is unknown. The primary objective of this study was to determine whether the induction of immune tolerance at one site evokes compartmentalized or, alternatively, pervasive immune tolerance, precluding de novo sensitization to the same Ag at distant mucosal and nonmucosal sites.

To address this question, we first comprehensively defined the site of tolerance induction at the level of secondary lymphoid organs, to determine which sites (and LNs) to subsequently target for Ag re-exposure. Previous studies have reported a vital role for CD4⁺ T cells in the induction of immune tolerance following intratracheal (15, 16), i.n. (48–52), aerosolized (18, 37, 53), oral (54, 55), or i.v. (56, 57) Ag delivery systems. From these studies, we reasoned that the site(s) in which CD4⁺ T cells engage cognate Ag and proliferate in vivo would most likely be indicative of the site in which T cell tolerance is programmed. By tracking CFSE-labeled donor transgenic T cells, we established that the induction of inhalation tolerance occurs selectively within the thoracic LNs in vivo, and importantly not in distant mucosal and nonmucosal LNs, or in the spleen. In other models of airway tolerance, most notably those using the i.n. route, the nose-draining cervical LNs appeared to be the predominant site for tolerance induction (48, 49). In models of orally fed Ag, both mesenteric LNs and Peyer's patches were shown to be the predominant sites of Ag presentation (58–61), and critical for the induction of oral tolerance (59, 62). To a lesser extent, oral Ag delivery has also been shown to induce clonal expansion of Ag-specific T cells in the spleen and, in cases in which high doses of Ag are administered, even in peripheral LNs (61, 63, 64). In our study, only very minute populations of donor transgenic T cells underwent proliferation in distal LNs. Moreover, because the adoptive transfer of transgenic T cells artificially heightens the frequency of Ag-specific clones, the minute proliferative events in distal LNs are likely to be nonexistent under

physiological conditions in which the frequency of Ag-specific T cells would be far less (<1/1000). Indeed, while this minute proliferation was observed, our data clearly demonstrated that the absolute number of these cells in distal LNs during Ag aerosolization was extremely small. A number of reports have proposed the lung itself to serve as a site of Ag presentation and primary T cell priming to live replicating Ags (influenza virus infection) through the induction of bronchus-associated lymphoid tissue (65, 66). However, the nature of the Ag is likely to be important, as we have previously shown that in mice devoid of LNs and spleen, the lung is unable to evoke Ag-specific primary immune responses to the model Ag, OVA (67).

Although inhaled Ag evoked a robust response under tolerizing conditions, it was significantly less than that observed under priming conditions. This intimates that the absolute number of Ag-specific CD4⁺ tolerizing T cells available to circulate throughout the periphery and mediate tolerance in vivo would be considerably less than the numbers of effector Th2 clones available to circulate throughout the periphery and mediate immunity in vivo. In agreement with this, we were unable to detect CFSE^{dim} Ag-specific donor transgenic T cells in the BAL compartment of tolerized mice, but were able to easily detect them in primed animals (data not shown). The number of activated OVA-specific T cells in tolerized animals would be expected to decline over time, leaving behind an even smaller, but stable, memory pool. This is consistent with previous studies that have shown a stable, but small, population of tolerizing T cells to persist in the periphery after the induction of immune tolerance (68). These T cells remained tolerogenic, as they were able to transfer tolerance to naive mice and protect against subsequent immunogenic Ag challenge. It is important to note that the transfer of tolerogenic activity is a most informative, but rather contrived experiment, in that it may not adequately represent what happens in vivo, and in the same animal. For example, the number of tolerizing CD4⁺ T cells required to transfer tolerance is usually in the order of 10^5 – 10^6 cells, which is clearly higher than the stable memory pool that persists following tolerance induction in vivo. It is for this reason that we asked whether this comparatively minute population of tolerizing memory T cells could effectively maintain a state of Ag unresponsiveness, in the same animal, upon subsequent Ag re-exposure at sites/LNs distant to the site of initial tolerance induction.

To test this hypothesis, tolerized mice were re-exposed to the same Ag at the same site (airways), under conditions conducive to the generation of robust Th2 sensitization. Consistent with our previous findings (69), Ag-specific proliferation and Th2

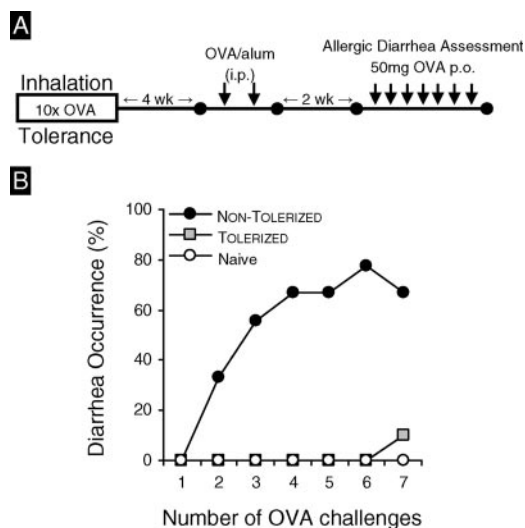


FIGURE 6. Tolerance induction via the respiratory mucosa prevents the development of Ag-induced allergic diarrhea. *A*, Time line illustrates mice were initially exposed to aerosolized OVA daily for 20 min over a period of 10 consecutive days (or left unexposed to Ag), and then rested for a 4-wk period before subjecting them to a protocol of i.p. Th2 sensitization to the same Ag (OVA). Two weeks post-i.p. Th2 sensitization, mice were orally administered 50 mg of OVA every other day and monitored for the development of allergic diarrhea. *B*, Results depict diarrhea occurrence (%; $n = 6$ –10 mice/group). Data are representative of three independent experiments.

differentiation in local draining LNs and Th2 immune inflammatory responses in the airway were prevented in mice previously tolerized through the respiratory mucosa. However, we considered that perhaps de novo Th2 sensitization was thwarted because the protocol of respiratory mucosal Th2 sensitization uses the same set of LNs used during inhalation tolerance. Hence, to adequately test the compartmentalization of immune tolerance, we examined whether prior respiratory mucosal tolerance would prevent de novo sensitization and effector responses to the same Ag if administered via the i.p. route. Our data demonstrate, unequivocally, that it did. However, while i.p. priming evoked Ag-specific T cell proliferation and Th2 differentiation in gut-associated Peyer's patches and mesenteric LNs, we and others have previously shown that the i.p. route drains primarily to the thoracic LNs (40, 44). Thus, although the site of Ag re-exposure (peritoneum) was in stark contrast to the site of initial Ag exposure (airways), Ag presentation occurred, to some degree, in the same LNs previously used during tolerance induction. Thus, we investigated whether the impact of tolerance induced through the respiratory mucosa extended to tissues/LNs both distant and nonrelated (i.e., nonmucosal) to the LNs involved in respiratory mucosal tolerance. To this end, tolerized mice were re-exposed to the same Ag, in an experimental system in which Ag was exclusively delivered to the skin via GG technology. Yet, in this experimental setting, the effects of respiratory mucosal tolerance still presided over events triggered in distant skin-draining LNs.

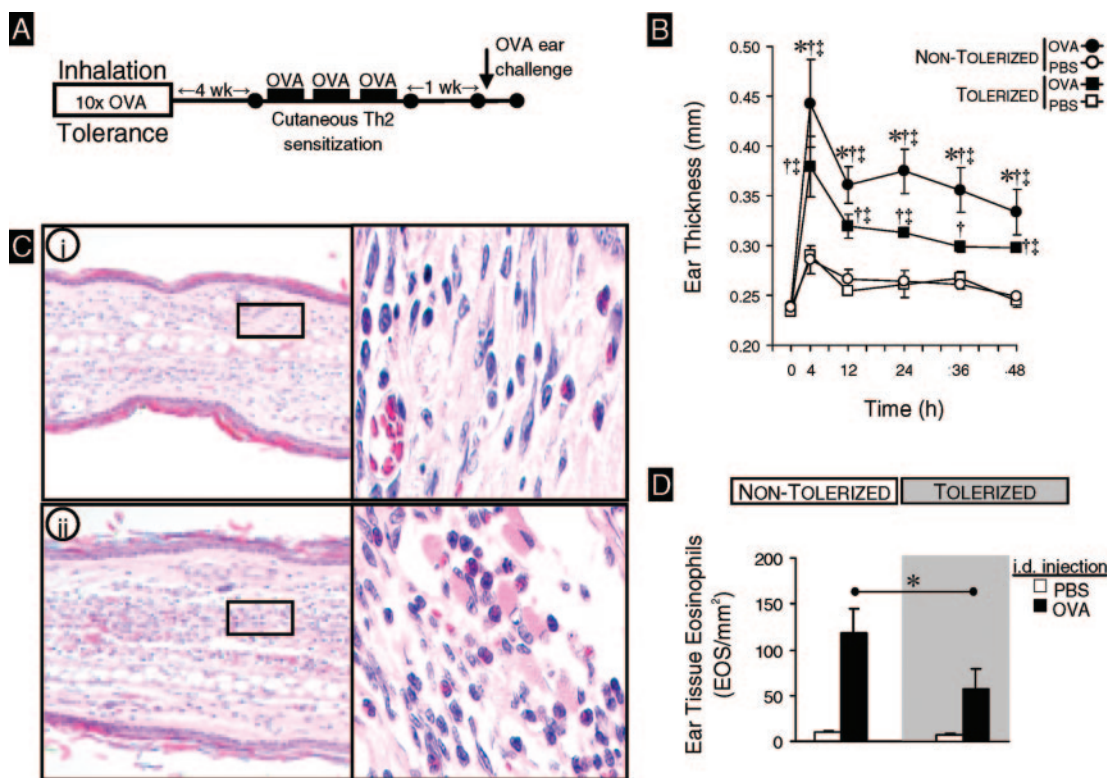


FIGURE 7. Tolerance induction via the respiratory mucosa inhibits late-phase cutaneous Th2 immune inflammatory responses. *A*, Time line illustrates mice were initially exposed to aerosolized OVA daily for 20 min over a period of 10 consecutive days (or left unexposed to Ag), and then rested for a 4-wk period before subjecting them to a protocol of cutaneous Th2 sensitization to the same Ag (OVA). One week postcutaneous Th2 sensitization, mice were injected with OVA protein into one ear and vehicle (PBS) into the opposite ear. *B*, Changes in ear thickness following OVA application (10 μ g i.d.) at the indicated time points. Statistical analysis was performed using one-way, repeated measures ANOVA with Fisher's least significant difference post hoc test (*, $p < 0.05$ compared with nontolerized/OVA i.d.; †, $p < 0.05$ compared with nontolerized/PBS i.d.; ‡, $p < 0.05$ compared with tolerized/PBS i.d.). *C*, Light photomicrographs of paraffin-embedded cross-sections of ear skin 48 h following OVA ear challenge in tolerized (*i*) and nontolerized (*ii*) mice. Original magnification of panels, $\times 100$. Magnification of insets, $\times 400$. *D*, Eosinophil infiltration into ear tissue following induction of late-phase cutaneous responses. Ear tissue eosinophils were enumerated 48 h following OVA ear challenge in nontolerized or tolerized mice ($n = 3$ –4/group). Data represent the mean $\pm$ SEM of eosinophil counts per mm^2 ear tissue. *, $p < 0.05$. Results are representative of at least two independent experiments.

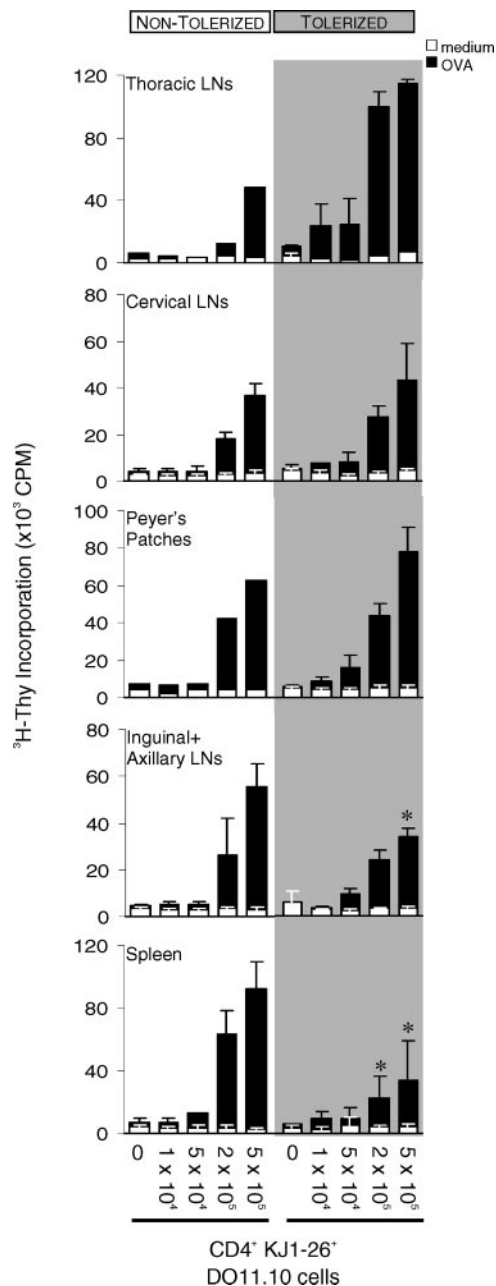


FIGURE 8. Suppressive activity resides in the systemic compartment. Thoracic LNs, cervical LNs, Peyer's patches, inguinal/axillary LNs, and spleen cells were harvested from mice undergoing respiratory mucosal tolerance to OVA (day 7 of protocol) or naive controls (pooled individually from five mice per group). Cells were stimulated in vitro with OVA or medium, in the presence of increasing concentrations of naive CD4⁺CD62L⁺ OVA-specific DO11.10 responder T cells. Ag-specific proliferation was assessed by [^3H]thymidine incorporation. Data are expressed as mean $\pm$ SD of triplicate wells, where applicable. *, $p < 0.05$, compared with nontolerized conditions by Student's t test.

Whereas a number of groups have investigated the consequences of inhalation or oral tolerance on secondary immunogenic challenge, for the most part these studies focused on immune-effector responses in various tissues (70, 71). For instance, Strid et al. (72) recently reported in a model of oral tolerance to peanut Ag that oral tolerance induction was able to inhibit delayed-type hypersensitivity responses after footpad challenge with peanut Ag. However, whether oral tolerance also inhibited Th2 differentiation in LNs draining the site of epicutaneous sensitization was not ex-

amined (72). Moreover, studies examining the effect of airway tolerance on inflammatory responses in the skin have done so by transferring tolerizing LN cells mixed with Th1 or Th2 effector cells directly into the site of Ag injection (15) and, thus, do not adequately address the issues explored in this manuscript, namely the compartmentalization of immune tolerance. In this study, we examined the impact of respiratory mucosal tolerance on cutaneous as well as GI mucosal responses, in an experimental system in which de novo sensitization specifically occurred in LNs distant to the site of tolerance induction, and in the same animal. We are the first to show that, despite the fact that the induction of a tolerogenic immune response occurs selectively within local draining LNs, the state of Ag unresponsiveness is pervasive (i.e., maintained at distant mucosal and nonmucosal LNs/tissues). In support of this pervasive tolerogenic phenotype, the induction of immune tolerance via the mesenteric LNs and Peyer's patches (i.e., oral tolerance) has also been shown to inhibit eosinophilic inflammation in the upper airways (73) and cellular-mediated cutaneous inflammation (74).

Collectively, these findings suggested that perhaps the tolerogenic activity induced during inhalation tolerance resided throughout the lymphatic system, as it has been shown for the central memory T cell subset evoked during immunity (75). However, even though the thoracic LNs were almost exclusively involved in tolerance induction, cells harvested from these LNs (and distal LNs) were unable to suppress the proliferative expansion of DO11.10 responder T cells in vitro. In contrast, we did detect potent suppressive activity in the spleens of mice following tolerance induction. The importance of the spleen in harboring the tolerogenic/suppressive phenotype is consistent with several adoptive transfer experiments demonstrating transferred tolerance with splenocyte-derived populations (13, 76, 77). Of note, there are studies that have shown tolerance to operate without suppressing proliferation (78); thus, it may be possible that the thoracic LNs do in fact harbor tolerogenic activity, but by a mechanism other than suppression. In support of this, LN-derived populations have been used to transfer tolerance to naive recipients, but either required substantially higher cell numbers or used activated/proliferating CD4⁺ T cells harvested from LNs actively undergoing tolerance induction and, importantly, not LNs that once harbored tolerance induction (15, 49). It may be possible that tolerized T cells are not imprinted with a tissue-selective tropism, but rather are capable of recirculating between mucosal and nonmucosal LNs/tissues. In this case, it will be of considerable importance to determine the identity of this tolerizing memory T cell subset in our system, largely, to determine the expression profile of a number of different homing molecules. Our initial attempts to follow the CFSE-labeled CD4⁺ donor transgenic T cell population following OVA aerosolization have been hampered, in part, by the reduced intensity of the CFSE label, TCR down-regulation, and the insufficient number of tolerizing T cells that remain within the memory pool to perform detailed flow cytometric analysis. However, it is promising that in an Ag delivery system identical with ours, Ostrokhova et al. (13) have recently identified an important role for the CD4⁺CD25⁺ FOXP3-expressing regulatory T cell subset in mediating immunosuppression; however, the homing receptor profile of this subset was not determined.

The pervasive protection induced following respiratory mucosal tolerance to allergens is at variance with the notion of tissue-selective immune-effector responses induced by the exposure of pathogens. Intuitively, tissue-selective immune memory is advantageous, as it concentrates the immune effort preferentially to sites once perturbed by pathogens. Our findings argue that, unlike immunity, immune tolerance to environmental Ags, and perhaps self

Ags, is not compartmentalized. In this context, pervasiveness is what may confer the biological advantage as it protects the body from generating immune inflammatory responses to innocuous Ags, responses that are not needed and can, in fact, be detrimental.

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Disclosures

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