

Cutaneous Antigen Priming via Gene Gun Leads to Skin-Selective Th2 Immune-Inflammatory Responses¹

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It is becoming increasingly evident that the compartmentalization of immune responses is governed, in part, by tissue-selective homing instructions imprinted during T cell differentiation. In the context of allergic diseases, the fact that “disease” primarily manifests in particular tissue sites, despite pervasive allergen exposure, supports this notion. However, whether the original site of Ag exposure distinctly privileges memory Th2 immune-inflammatory responses to the same site, while sparing remote tissue compartments, remains to be fully investigated. We examined whether skin-targeted delivery of plasmid DNA encoding OVA via gene-gun technology in mice could generate allergic sensitization and give rise to Th2 effector responses in the skin as well as in the lung upon subsequent Ag encounter. Our data show that cutaneous Ag priming induced OVA-specific serum IgE and IgG1, robust Th2-cytokine production, and late-phase cutaneous responses and systemic anaphylactic shock upon skin and systemic Ag recall, respectively. However, repeated respiratory exposure to aerosolized OVA failed to instigate airway inflammatory responses in cutaneous Ag-primed mice, but not in mice initially sensitized to OVA via the respiratory mucosa. Importantly, these contrasting airway memory responses correlated with the occurrence of Th2 differentiation events at anatomically separate sites: indeed cutaneous Ag priming resulted in Ag-specific proliferative responses and Th2 differentiation in skin-, but not thoracic-, draining lymph nodes. These data indicate that Ag exposure to the skin leads to Th2 differentiation within skin-draining lymph nodes and subsequent Th2 immunity that is selectively manifested in the skin. *The Journal of Immunology*, 2005, 174: 1664–1674.

The skin and respiratory mucosae are continuously exposed to the external environment and, thus, represent major entry points for infection. To provide effective immune surveillance, the immune system has evolved trafficking patterns that allow homing of particular lymphocyte subsets to specific tissue sites (1). In this regard, memory/effector T cells have been identified that selectively home to cutaneous (2–4) and various mucosal (5–7) surfaces. Moreover, there is accumulating evidence that imprinting (8–10) or selection (10) for differential homing instructions including, but not limited to, adhesion molecules (5, 7, 11) and chemoattractant receptors (12–16) form an integral part in the establishment of a tissue-selective trafficking pattern for memory T lymphocytes. Likewise, the discovery of several epithelial-derived and tissue-restricted chemokines in cutaneous (17) and diverse mucosal sites (18–21) also intimates an important role for local tissue-derived signals in preferentially directing and retaining specific T lymphocyte subsets.

Although the immune system may favor compartmentalization of the inflammatory response to the site of Ag encounter, it may also be advantageous that at least some elements of the immune response remain site-unrestricted to allow protection against future encounters with the same Ag at different sites. These issues are not only important to the development of vaccination strategies, but also to our understanding of the clinical manifestations of noninfectious, immunologically driven processes such as allergy. Indeed, the natural history of allergic disease presents with distinct organ manifestations, such as in the skin (atopic dermatitis) and the respiratory tract (allergic asthma), which often develop sequentially in time, but not always in the same individual (22–25). Whether the site of initial Ag exposure determines the long-term clinical expression of allergic disease remains to be fully elucidated. Investigation of this issue requires a system that exquisitely confines Ag delivery to one compartment during initial Ag sensitization. In this regard, particle bombardment of Ag DNA through gene-gun (GG)³ technology strictly restricts the Ag to the epidermal/dermal layers (26–29). That GG-mediated Ag delivery to the skin also biases the ensuing immune response toward Th2 (30–32), makes this technology particularly suitable to study aspects of the compartmentalization of allergic Th2 responses.

Here, we have investigated whether the initial site of Ag exposure evokes site-restricted Th2 immune memory in an experimental paradigm in which Ag is exclusively delivered to the skin via GG technology. To this end, we have comprehensively investigated the ensuing Th2 immune-inflammatory recall response at

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Received for publication August 26, 2004. Accepted for publication October 13, 2004.

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¹ This research was supported by the Canadian Institutes for Health Research. We also recognize the support of the Hamilton Health Sciences Corporation and St. Joseph's Hospital (Hamilton, Ontario, Canada). D.A. holds a Doctoral Research Award from the Canadian Institutes for Health Research. R.F. holds a graduate scholarship from National Sciences and Engineering Research Council of Canada.

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³ Abbreviations used in this paper: GG, gene-gun; Ad, adenoviral; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; e.c., epicutaneous; i.d., intradermal; LN, lymph node; MCh, methacholine; MHCII, MHC class II; MNC, mononuclear cell; PB, peripheral blood; RRS, respiratory system resistance; SAS, systemic anaphylactic shock; TARC, thymus- and activation-regulated chemokine.

both the original site of Ag exposure and a distant mucosal compartment. Our data show that cutaneous Ag priming through repeated GG delivery of plasmid DNA encoding OVA generated Ag-specific Th2-associated Igs and cytokines and provoked Th2-effector responses, namely systemic anaphylactic shock (SAS) and late-phase cutaneous responses, upon systemic and skin Ag recall, respectively. However, respiratory OVA exposure failed to elicit Th2-effector responses in the lung of cutaneous Ag-primed mice, but not in mice initially mucosally sensitized to OVA via the airways. Examination of secondary lymphoid organs revealed Ag-specific proliferative responses and Th2 differentiation solely in skin-, but not thoracic-, draining lymph nodes (LNs) following GG cutaneous priming. These data demonstrate that cutaneous Ag priming preferentially restricts Th2 immune-inflammatory memory responses to the skin, and insinuate that this restriction is acquired in the draining LNs where T cell differentiation occurs.

Materials and Methods

Animals

Female BALB/c 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. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

GG specifications

The Helios gene-gun (Bio-Rad Laboratories) was used to deliver plasmid DNA onto the skin. Three plasmids were used for these studies: *pOVA*-003, *pLUC*-003, and *pCMV*, containing the cDNAs for OVA, firefly luciferase, and an empty cassette, respectively, under control of the human CMV intermediate-early promoter. The plasmid backbone for all these studies was a modified version of *pcDNA3.1+* (Invitrogen Life Technologies), where the neomycin resistance cassette was removed. Plasmids were prepared using Qiagen Endo-Free Mega Prep kits (Qiagen) and resuspended at a final concentration of 3 $\mu\text{g}/\mu\text{l}$ in endotoxin-free, sterile water (Invitrogen Life Technologies). Preparation of DNA-coated gold particles was performed according to manufacturer specifications. Briefly, each plasmid DNA construct (0.01–1 μg) was added to 15 mg of 1.6- μm gold particles (Bio-Rad) in 0.05 M spermidine (Sigma-Aldrich). Next, 200 μl of 1 M CaCl_2 was added dropwise to the mixture with gentle vortexing and was allowed to precipitate for 10 min at room temperature. Gold pellets were washed three times in 100% ethanol and resuspended in ethanol solution containing polyvinylpyrrolidone (0.05 mg/ml; Bio-Rad). Finally, the DNA-gold suspension was loaded and dried onto the surface of Tefzel tubing (Bio-Rad) by N_2 gas, then cut into cartridges and stored desiccated at 4°C until required.

Cutaneous Ag priming

Cutaneous priming to OVA was achieved through GG bombardment of plasmid OVA-coated gold particles onto the abdominal epidermis of mice. Mice were anesthetized with isoflurane, and the ventral abdominal surface was shaven and wiped clean with antiseptic isopropyl alcohol pads. Each animal received three nonoverlapping deliveries of DNA-coated gold particles, totaling 0.01–1 μg of DNA per inoculation (i.e., 0.0033–0.33 μg of DNA per delivery). The end-barrel of the GG was held directly against the abdominal skin, and DNA-coated particles were discharged at a helium pressure of 350 lb/in². Mice were either inoculated once (day 0) or on three occasions at weekly intervals (days 0, 7, and 14) with gold alone, *pLUC*, *pCMV*, or *pOVA*.

Respiratory mucosal priming

Respiratory mucosal priming to OVA was induced via the airways as previously described (33). Briefly, mice were exposed to aerosolized OVA in the context of a GM-CSF-enriched airway microenvironment conducive to the generation of allergic sensitization. A replication-deficient human type 5 adenoviral (Ad) construct encoding murine GM-CSF cDNA in the E1 region of the viral genome (Ad/GM-CSF; Ref. 34) was delivered intranasally to isoflurane-anesthetized mice on day –1. The Ad/GM-CSF vector was administered at a dose of 3×10^7 PFU in 30 μl of PBS. Over a period of 10 consecutive days (days 0–9), mice were placed in a Plexiglass chamber (10 cm $\times$ 15 cm $\times$ 25 cm) and exposed for 20 min daily to aerosolized OVA (grade V protein, 1% w/v in 0.9% saline; Sigma-Aldrich). The OVA aerosol was generated by a Bennet/Twin nebulizer at a flow rate of 10

L/min. Respiratory mucosally primed mice were allowed to recover from the acute airway inflammation (~3–4 wk) before being re-exposed to aerosolized OVA (~day 35) as part of the Ag recall experiments outlined below.

Splenocyte and LN cell culture

Spleens or LNs (axillary, inguinal, or mediastinal/thoracic) were excised at various time points during cutaneous or respiratory mucosal priming 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 70- μm 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 ACK lysis buffer (0.5 M NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA at pH 7.2–7.4) for 1 min. 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 μg 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 μCi /well of [³H]thymidine (PerkinElmer Life Sciences, Boston, MA) was added to the last 18 h of a 3-day culture, in the presence or absence of OVA (40 μg /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 $\pm$ SEM of triplicate wells.

Flow cytometric analysis

Isolated LN cells were washed twice in FACS buffer (0.5% BSA in PBS) and then stained. To minimize nonspecific binding, cells were preincubated with FcBlock (BD Pharmingen). The following Abs and reagents were used: mouse IgG3 anti-mouse MHC class II (MHCII; I-A^d), FITC-conjugated (39-10-8); mouse IgG3 isotype control, FITC-conjugated (A112-3); hamster IgG anti-mouse CD69, PE-conjugated (H1.2F3); hamster IgG isotype control, PE-conjugated (G235-2356); anti-mouse CD3 ϵ , CyChrome-conjugated (145-2C11); anti-mouse CD4, PE $\cdot$ Cy7-conjugated (RM4-5); anti-mouse CD8, APC-conjugated (53-6.7); anti-mouse B220, APC-conjugated (RA3-6B2); anti-mouse CD19, biotinylated (1D3); streptavidin-PE $\cdot$ Cy7 (all purchased from BD Pharmingen). The Abs were titrated to determine optimal concentration. For each Ab combination, 1×10^6 cells were incubated with mAbs for 30 min on ice, 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 (Scripps Research Institute). A total of 100,000–200,000 events were acquired.

Late-phase cutaneous responses

Late-phase cutaneous responses were induced in mice by intradermal (i.d.) injection with 10 μg of OVA in 10 μl of sterile saline into one ear, and vehicle (saline) into the opposite ear, 1 wk after cutaneous Ag priming and in naive controls. Ear thickness was measured before and at several time points after injection using a pocket thickness gauge (Mitutoyo) equipped with a preloaded spindle to provide a constant closing force of <0.2 Newtons. Mice were sacrificed 36 h later, and ears were fixed in 10% formalin, 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.

Systemic anaphylactic shock

SAS was induced by i.v. injection with 100 μg of OVA in 100 μl of PBS into the tail vein of cutaneous Ag-primed or naive mice. The mice were carefully observed for 40 min immediately following injection. Core body temperature readings were performed every 10 min with a rectal probe digital thermometer (VWR). The anaphylactic reaction was evaluated and rated according to a modified SAS scoring system (35). SAS legend: 0, no sign; 1, decreased activity and piloerection; 2, loss of coordination and dyspnea; 3, no response to whisker stimuli and only slight response to prodding; 4, no response to tail pinch and progressive paresis; 5, convulsion, prostration, or coma. In concordance with guidelines set by the Canadian Council on Animal Care and our institution, moribundity was avoided as an endpoint and euthanasia was conducted if SAS scores exceeded four. Plasma histamine levels were determined at the end of the

40-min observation period using an immunoassay kit (IBL) following the manufacturer's specifications.

Aerosolized OVA recall to the lung

Mice initially sensitized to OVA through the skin or respiratory mucosa (or unsensitized control mice), were challenged with repeated respiratory exposure to aerosolized OVA, ~3–4 wk after priming. Specifically, skin- or respiratory mucosal-sensitized mice were exposed to 1% OVA aerosol daily for 20 min for 1, 3, or 5 consecutive days. Unsensitized and unchallenged naive mice served as negative controls. In all experiments, mice were sacrificed 72 h following the last OVA aerosolization, and the ensuing immune response in the airway was assessed as detailed below.

Collection and measurement of specimens

Following OVA aerosolization mice were sacrificed and peripheral blood (PB) collected by retro-orbital bleeding. PB smears were prepared, and total white blood cell counts were determined in a blinded manner using a hemocytometer. Serum was obtained by centrifugation after incubating whole blood for 30 min at 37°C; serum was stored as aliquots at -20°C. In addition, bronchoalveolar lavage (BAL) was performed as previously described (36). 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. PB and BAL smears were stained with the Protocol Hema 3 stain set (Fischer Scientific). Differential cell counts of PB and BAL smears were determined in a blinded manner from at least 300–500 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells (MNC). 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 PAS (for detection of goblet cells).

Assessment of airway physiology

Airway hyperresponsiveness (AHR) was assessed 72 h following the fifth OVA aerosolization in skin-sensitized, respiratory mucosal-sensitized, or unsensitized naive control mice. Airway responsiveness was determined on the basis of the response of total respiratory system resistance (RRS) to increasing i.v. (internal jugular vein) doses of methacholine (MCh) as previously described (37). Briefly, mice were anesthetized with tribromoethanol (287 mg/kg i.p.) prepared according to a standard protocol (38). The

trachea was surgically exposed and cannulated, and a constant inspiratory flow was delivered by mechanical ventilation (RV5; Voltek Enterprises). Heart rate and oxygen saturation were monitored via infrared pulse oxymetry (Biox 3700; Ohmeda), using a standard ear probe placed over the proximal portion of the hind limb. Paralysis was achieved using pancuronium (0.03 mg/kg i.v.) to prevent respiratory effort during measurement. RRS was measured after consecutive i.v. injections of saline, then 10, 33, 100, and 330 μ g/kg of MCh (ACIC), each delivered as a 0.2-ml bolus. Evaluation of AHR was based on the peak RRS measured in the first 30 s following the saline and MCh doses.

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), Eotaxin, MIP-1 α , and RANTES. Each of these assays has a threshold of detection between 1.5 and 5 pg/ml. In some instances, IL-4, IL-5, IL-10, and IL-13 content was determined using Beadlyte mouse multicytokine fluorescent bead-based FLEX assays (Upstate Biotechnology) as previously described (39), and quantified using a Luminex¹⁰⁰ Instrument (Luminex) according to manufacturer's instructions. Levels of OVA-specific serum IgE were measured using a previously described Ag-capture (biotinylated OVA) ELISA method (36). OVA-specific serum IgG1 was measured by sandwich ELISA with OVA in the solid phase, as previously described in detail (40). Units of OVA-specific IgE were determined relative to in-house standardized serum, obtained from mice sensitized to OVA through a conventional i.p. sensitization model for IgE and IgG1 standards (36). Sample Ig levels are expressed in units per milliliter relative to standard mouse sera.

Data analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using SigmaStat software (SPSS). Results were interpreted using ANOVA followed by Fischer's least significant difference post hoc test analysis, unless otherwise indicated. A *p* value of <0.05 was considered statistically significant.

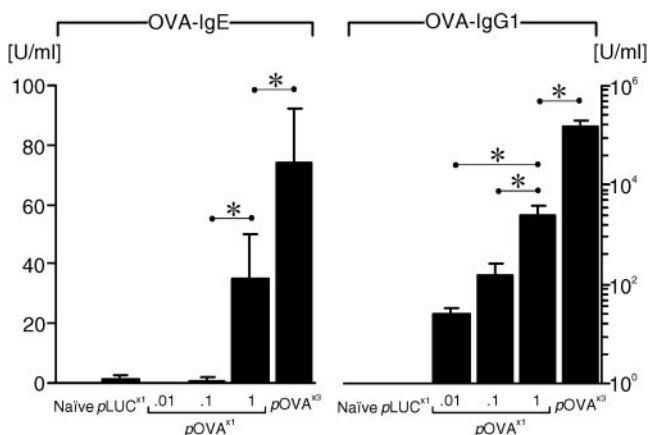


FIGURE 1. Cutaneous Ag priming via GG induces Ag-specific, Th2-associated serum Ig production in mice. Mice were primed with Ag via the skin by way of GG-mediated particle bombardment of gold particles coated with Ag-encoded plasmids. The graph depicts serum levels (mean $\pm$ SEM) of OVA-specific IgE and IgG1 following single ($\times 1$) or repeated ($\times 3$) GG delivery of (0.01, 0.1, or 1 μ g) pOVA or irrelevant Ag control plasmid (pLUC). Igs are expressed as units per milliliter and were measured by ELISA; *n* = 3 per group, and representative of two independent experiments; *, *p* < 0.05.

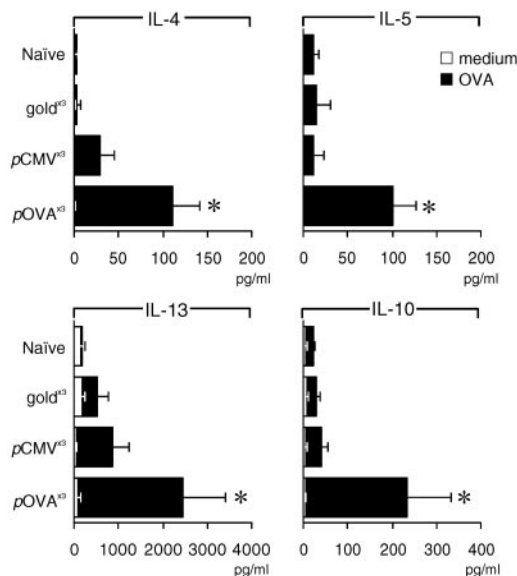


FIGURE 2. Cutaneous Ag priming induces Ag-specific, Th2-cytokine production by cultured splenocytes. Splenocytes were harvested 1 wk following the third cutaneous GG particle bombardment and stimulated for 120 h with OVA or medium alone in vitro. Graph depicts IL-4, -5, -10, and -13 production by cultured splenocytes. The bars represent unsensitized mice (naive) and mice bombarded on three occasions with gold alone (gold $\times 3$), empty-control plasmid (pCMV $\times 3$), or plasmid encoding OVA (pOVA $\times 3$). Data represent mean $\pm$ SEM; *n* = 4–7 per group, and representative of two independent experiments; *, *p* < 0.05 compared with all groups.

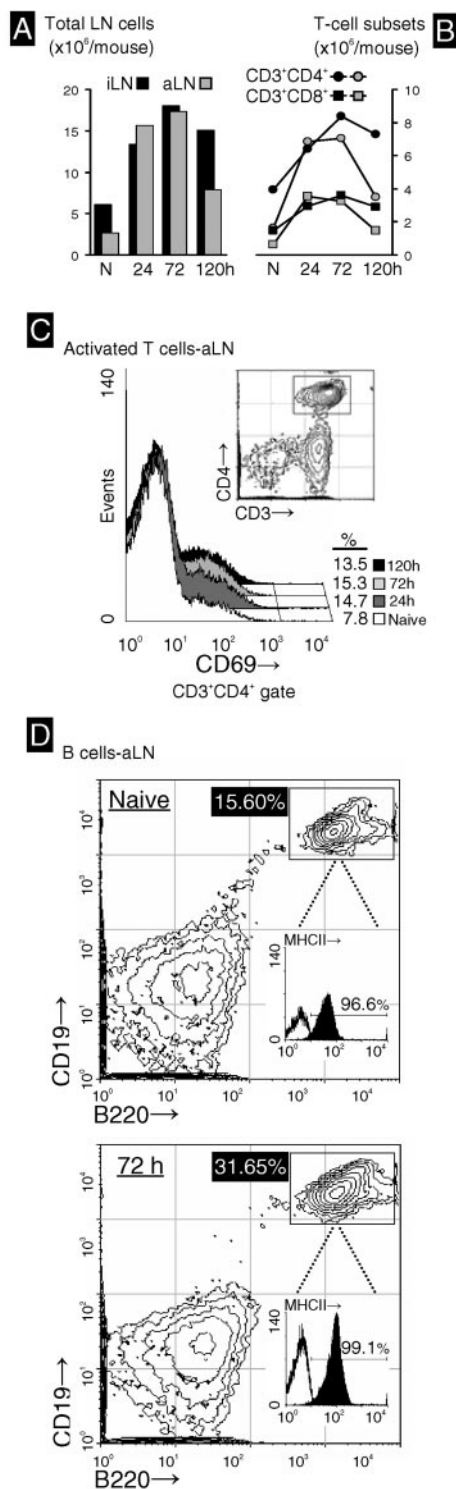


FIGURE 3. Flow cytometric analysis of lymphocyte populations in skin-draining LNs following cutaneous Ag priming. At the indicated time points following cutaneous priming, mice were sacrificed, and two sets of skin-draining LNs (axillary (aLN) and inguinal (iLN)) removed and pooled separately from four mice for flow cytometric analysis. Data indicate the total number of nucleated LN cells ($\times 10^6/\text{mouse}$) (A) and the absolute number of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells after priming (B). C, Activation status (i.e., CD69 expression) of gated CD3⁺CD4⁺ T cells 72 h after priming vs naive controls. D, Marked increase in MHCII-expressing CD19⁺B220⁺ B cells 72 h after priming compared with naive controls. Data in C and D depict activation status and B cell expansion for axillary LNs, with similar results obtained from inguinal LNs. All analysis is based on events collected in the mononuclear/lymphocyte gate. Data in A and B are representative of three independent experiments, and C and D of two independent experiments.

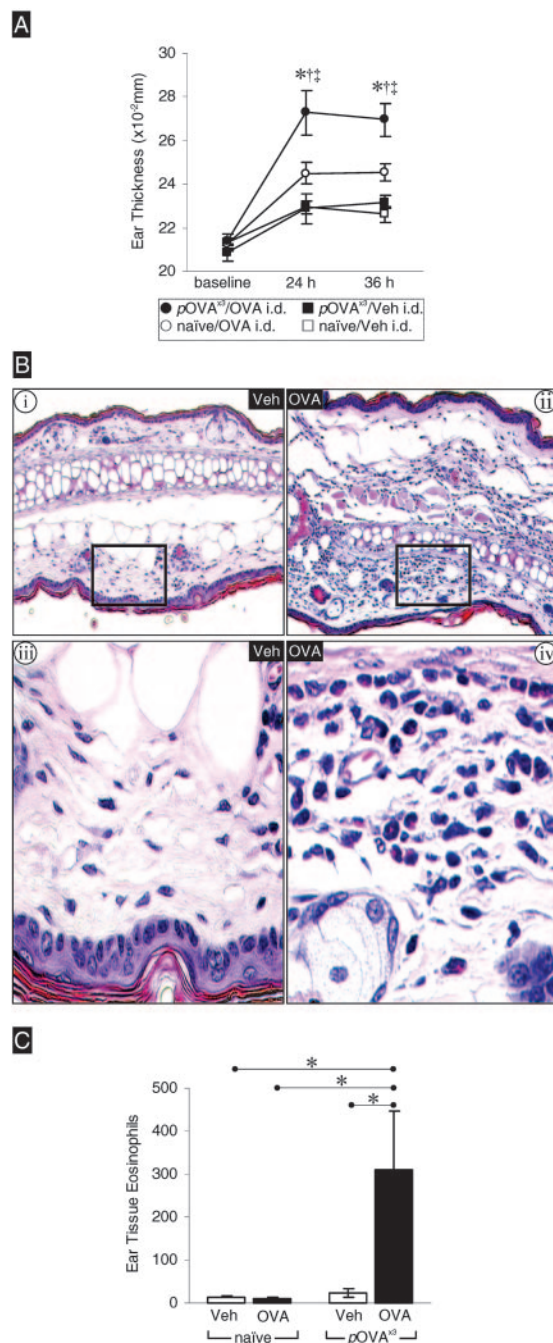


FIGURE 4. Ag administration to the skin evokes late-phase cutaneous inflammatory responses in cutaneous-primed mice. One week after cutaneous priming, pOVA^{Δ3}-primed mice (or naive controls) were injected i.d. with OVA protein into one ear and vehicle (saline) into the opposite ear, and monitored for changes in ear thickness (A) at the indicated time points. Statistical analysis was performed using one-way repeated measures ANOVA with Newman-Keuls post hoc test (*, $p < 0.05$ compared with naive/OVA i.d.; †, $p < 0.05$ compared with naive/Veh i.d.; ‡, $p < 0.05$ compared with pOVA^{Δ3}/Veh i.d.). B, Light photomicrographs of paraffin-embedded cross-sections of ear skin 36 h following i.d. OVA recall in vivo. All panels (i, ii, iii, and iv) represent sections obtained from pOVA^{Δ3}-primed mice stained with H&E. i and iii represent sections following i.d. ear injection with vehicle, whereas ii and iv represent sections following i.d. ear injection with OVA. Original magnification of panels, $\times 100$ (i and ii) and $\times 400$ (iii and iv). C, Eosinophil infiltration into ear tissue following induction of late-phase cutaneous responses. Ear tissue eosinophils were enumerated 36 h following cutaneous OVA recall in pOVA^{Δ3}-primed mice or naive controls. Data represent the mean $\pm$ SEM of eosinophil counts from each OVA (or vehicle) injected ear. $n = 3\text{--}4$ per group; *, $p < 0.05$.

Results

Induction of Ag-specific, Th2 immunity via cutaneous Ag priming

To assess whether Ag exposure to the skin generated Th2 sensitization, mice were immunized with plasmid encoding OVA cDNA via GG particle bombardment, and the nature of the ensuing immune response was comprehensively characterized. Single immunization via the skin with microgram quantities of $pOVA^{\times 1}$, but not with luciferase ($pLUC^{\times 1}$) or naive controls, resulted in the generation of OVA-specific serum IgE and IgG1 for 2 wk after immunization (Fig. 1). We also observed a dose-dependent increase in the levels of IgE and IgG1 with the highest Ig titer achieved following 1 μ g of plasmid delivery. Next, we increased the frequency of GG plasmid delivery from one to three immunizations, at weekly intervals, using the 1- μ g plasmid dose. We found that repeated delivery of $pOVA^{\times 3}$ significantly potentiated OVA-specific serum IgE and IgG1 levels, compared with a single immunization. Moreover, OVA-specific IgE and IgG1 levels were not induced following repeated immunization with gold alone or empty control plasmid (data not shown). It is important to note that serum levels of OVA-specific IgG2a, a Th1-affiliated Ig, were not significantly elevated following single or repeated $pOVA$ immunization, at any of the time points examined (data not shown).

That cutaneous Ag priming via GG led to the generation of Th2-associated Igs, prompted us to investigate other key markers of Th2 immunity. To this end, we harvested splenocytes 1 wk after cutaneous Ag priming and subsequently measured the production of a number of Th2-affiliated cytokines upon stimulation with either OVA or medium alone in vitro. Despite the detection of Th2-associated Igs (Fig. 1) following a single $pOVA^{\times 1}$ immunization, very low levels of IL-4 and IL-5 were detected from stimulated splenocytes (data not shown); hence, we report only cytokines produced by harvested splenocytes from mice repeatedly immunized with 1 μ g of $pOVA^{\times 3}$. Indeed, $pOVA^{\times 3}$ immunization resulted in robust production of IL-4, IL-5, IL-10, and IL-13 in vitro by OVA-stimulated splenocytes, compared with mice immunized with gold alone (gold $^{\times 3}$), an empty plasmid ($pCMV^{\times 3}$) or naive controls (Fig. 2). A similar Th2-polarized cytokine profile was induced following in vitro OVA stimulation of skin-draining LN cells following $pOVA^{\times 3}$ priming (see Fig. 8B). In either case, increasing the plasmid dose from 1 to 7.5 μ g did not significantly enhance cytokine production by splenocytes or LN cells (data not shown). Consistent with a lack of serum IgG2a production, we also observed no significant increase in IFN- γ by OVA-stimulated splenocytes or LN cells from $pOVA^{\times 3}$ -immunized mice (data not shown).

Lymphocyte expansion in skin-draining LNs following cutaneous Ag priming

Next, we examined different lymphocyte subsets by flow cytometric analysis in peripheral/skin-draining LNs at various time points

during cutaneous Ag priming. We consistently observed a pronounced cellular expansion of skin-draining inguinal and axillary LNs (Fig. 3A), and to a lesser extent brachial LNs (data not shown), within the first 5 days after cutaneous Ag priming. Peak LN expansion occurred 72 h after $pOVA^{\times 3}$ priming, with a 3- to 6-fold increase in total LN cells compared with naive LNs, respectively. We also observed an expansion in the absolute number of both CD3 $^{+}$ CD4 $^{+}$ and CD3 $^{+}$ CD8 $^{+}$ lymphocyte subsets (Fig. 3B), including a near 2-fold increase in expression of the early activation marker CD69 on gated CD3 $^{+}$ CD4 $^{+}$ T cells in axillary (Fig. 3C) and inguinal (data not shown) LNs compared with naive mice. Moreover, we also observed a greater than 2-fold increase in the proportion and absolute number of MHCII-expressing CD19 $^{+}$ B220 $^{+}$ B cells in skin-draining axillary (Fig. 3D) and inguinal (data not shown) LNs 72 h after $pOVA^{\times 3}$ priming compared with naive mice. Taken together, these data indicate that cutaneous Ag priming leads to immune activation of T and B lymphocytes in local skin-draining LNs.

Impact of cutaneous and systemic Ag challenge, in cutaneous Ag-primed mice

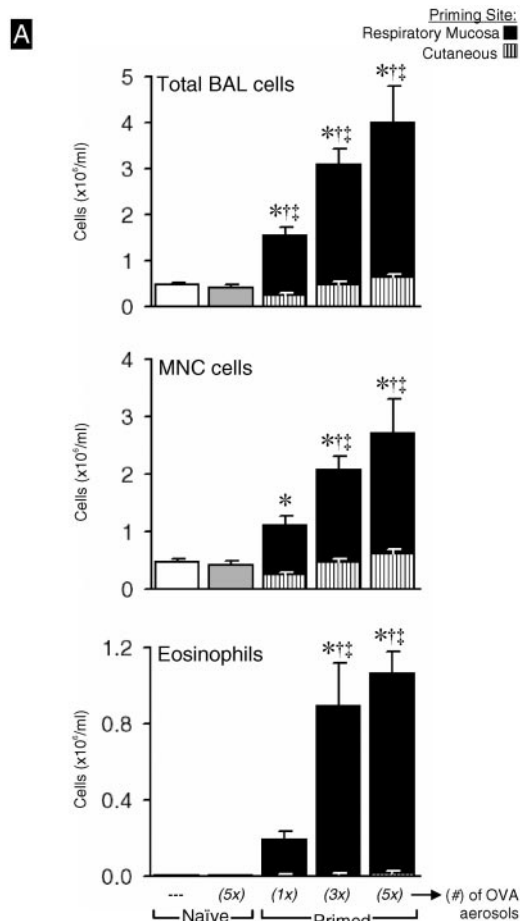
Next, whereas skin-targeted Ag DNA delivery induced Th2-associated Igs and cytokines, we assessed whether it could also give rise to allergic manifestations upon Ag re-exposure in vivo. To this end, we examined the impact of cutaneous and systemic re-exposure to the sensitizing Ag. Mice were repeatedly immunized at weekly intervals with $pOVA$ into the skin via GG, and 1 wk later injected i.d. with OVA or vehicle (saline) into the right and left ears, respectively. OVA, but not vehicle, injection led to a significant late-phase cutaneous response as assessed by an increase in ear thickness at 24 h and 36 h compared with baseline, and naive controls (Fig. 4A). Histological examination of ear tissue 36 h following i.d. OVA, but not vehicle, injection revealed a pronounced mononuclear and eosinophilic infiltrate (Fig. 4B). Indeed, enumeration of ear tissue eosinophils (Fig. 4C) was in agreement with histological findings, demonstrating a marked increase in infiltrating eosinophils in ears recalled with OVA but not vehicle. In another series of experiments, we injected OVA protein i.v. into naive or $pOVA^{\times 3}$ -skin-sensitized mice and carefully monitored mice for signs of SAS, as previously described (35). Systemic OVA injection into skin-sensitized, but not naive controls, resulted in abrupt decreased bouts of spontaneous activity and intermittent shivering that gradually worsened to no response to whisker stimuli and only slight reaction to prodding (Table I). Moreover, mice experienced a marked drop ($\sim 30\%$) in body temperature during the 40 min observation period following i.v. injection. This SAS response was also accompanied by a near 20-fold increase in plasma histamine levels in cutaneous Ag-primed mice following i.v. OVA administration compared with PBS injection.

Table I. Induction of SAS following systemic (i.v.) Ag exposure in cutaneous Ag-primed mice^a

Group	i.v.	Body Temperature (°C)					Histamine (ng/ml)	SAS scores
		0	10	20	30	40 min		
Naïve	PBS	37	35	37	37	37	9.6 $\pm$ 1.9	0,0,0,0,0
	OVA	37	37	37	38	37	17.3 $\pm$ 6.2	0,0,0,0,0
$pOVA^{\times 3}$	PBS	37	36	36	36	36	68.6 $\pm$ 31.8	0,0,0,0,0
	OVA	37	31 ^b	30 [*]	28 [*]	27 [*]	>1250	3,3,4,3,3

^a One week after cutaneous Ag priming via GG, mice were injected (i.v.) with either PBS vehicle or OVA (100 μ g) and monitored for the development of symptoms indicative of SAS. Body temperature was recorded every 10 min ($n = 6$), at which time mice were monitored and scored for the severity of SAS symptoms by multiple observers. Histamine levels were determined at the end of 40 min of observation. Results are representative of two independent experiments. See *Materials and Methods* for SAS score legend.

^b *, $p < 0.05$ compared with all groups.



	OVA Aerosolization:	
	Cutaneous priming	Respiratory Mucosal priming
BAL Cytokines		
IL-5	4.4 ± 4.4	36.6 ± 2.6 *
IL-13	<1.5	52.9 ± 11.1 *
BAL Chemokines		
MIP-1	3.6 ± 3.6	15.9 ± 7.9 NS
RANTES	7.0 ± 0.9	48.3 ± 13.5 *
TARC	3.6 ± 3.6	290.8 ± 73.1 *
Eotaxin	1.9 ± 1.9	11.2 ± 3.0 *

FIGURE 5. Respiratory exposure to aerosolized Ag fails to instigate allergic airways inflammatory responses in cutaneous Ag-primed mice, but does so in mice initially primed to Ag via the respiratory mucosa. Mice were initially sensitized to Ag via the skin by way of cutaneous GG-mediated particle bombardment (▨) or the airways via a well-characterized model of respiratory mucosal sensitization (■; Ref. 33), and 3–4 wk later were exposed to aerosolized OVA. Mice were exposed to either one (1×), three (3×), or five (5×) OVA aerosols then sacrificed 72 h after the last OVA aerosolization, and the number of total cells, MNC cells, and eosinophils in BAL (A) were determined. Unsensitized-unchallenged and unsensitized-OVA-challenged mice served as negative controls. *, $p < 0.05$ compared with unsensitized-unchallenged; †, $p < 0.05$ compared with unsensitized-OVA-challenged; ‡, $p < 0.05$ compared with cutaneous Ag-primed mice. B, Chemokine and cytokine levels in BAL fluid were determined 72 h after a single OVA aerosolization in mice previously primed to Ag via cutaneous or respiratory mucosal routes. Chemokines and cytokines were measured by ELISA. *, $p < 0.05$ compared with cutaneous-primed mice. Data in A and B are expressed as mean ± SEM; $n = 3$ –4 per group and representative of two independent experiments.

Impact of respiratory exposure to aerosolized OVA in cutaneous Ag-primed mice

We next investigated whether expression of Th2 immunity was site-restricted. Because Ag delivery to the skin induced local and systemic Th2 immunity resulting in skin and systemic allergic responses upon cutaneous and i.v. Ag re-exposure in vivo, we examined whether this route of sensitization could permit allergic manifestations at distant compartments such as the respiratory mucosa. To this end, mice were repeatedly skin-immunized to pOVA³ via the GG, and 3 wk later exposed to a 1% OVA aerosol for 20 min daily, for up to 5 consecutive days. Mice were sacrificed 72 h following the final OVA exposure, and the inflammatory infiltrate in the BAL assessed. Our previous experience with long-term Ag recall experiments in mice sensitized, either via a conventional i.p. model (36) or a model of respiratory mucosal sensitization (33), established that as little as one OVA exposure was able to evoke airway eosinophilic inflammation (D. Alvarez and M. Jordana, unpublished data). Contrary to this, even up to five consecutive OVA aerosolizations failed to provoke airway eosinophilic inflammation (Fig. 5A) in skin-sensitized mice. In fact, the response to OVA aerosolization in cutaneous Ag-primed resembled that of OVA challenged unsensitized control mice. Importantly, only mice that were initially mucosally sensitized in the context of a GM-CSF-enriched airway microenvironment (i.e., respiratory mucosal priming), were fully able to mount a robust airways inflammatory response, even after a single OVA aerosolization (Fig. 5A). The airway inflammatory infiltrate in these mice was characterized by a predominant increase in BAL MNCs (~67% of total BAL cells; Fig. 5A), eosinophils (up to ~30%; Fig. 5A), and a slight increase in BAL neutrophils (~3–5%; data not shown). In addition, OVA aerosolization led to a significant increase in Th2-associated cytokines (IL-5 and IL-13) and chemokines (TARC, RANTES, Eotaxin) in BAL fluid, in respiratory mucosal-, but not cutaneously, primed mice (Fig. 5B). It should be noted that levels of immunoregulatory (IL-10) or Th1 (IL-12, IFN-γ)-associated cytokines were not increased in the BAL of cutaneous Ag-primed mice upon OVA aerosolization, and thus not supporting the elaboration of a competing Th1 or regulatory immune response (data not shown). Consistent with the BAL findings, we

Table II. Peripheral blood leukocytes, serum Ig levels, and in vitro splenocyte cytokine production following repeated OVA aerosolization in cutaneous Ag-primed or naïve mice^a

	OVA Aerosolization	
	Naïve	Cutaneous Ag priming
PB leukocytes^b		
Total cells	73 ± 5	58 ± 14
MNC cells	60 ± 5	52 ± 12
Eosinophils	2.2 ± 0.3	2.6 ± 0.9
OVA-specific serum Igs^c		
IgE	15 ± 5	514 ± 101* ^d
IgG1 (×10 ³)	0.04 ± 0.02	565 ± 44*
In vitro cytokine production^e		
IL-4	4 ± 4	414 ± 70*
IL-5	0	1763 ± 409*
IL-13	332 ± 127	5945 ± 837*

^a Groups of mice were sacrificed 72 h following the fifth OVA aerosolization.

^b Differential cell analysis was performed on PB smears and expressed as ×10⁵ cells/ml.

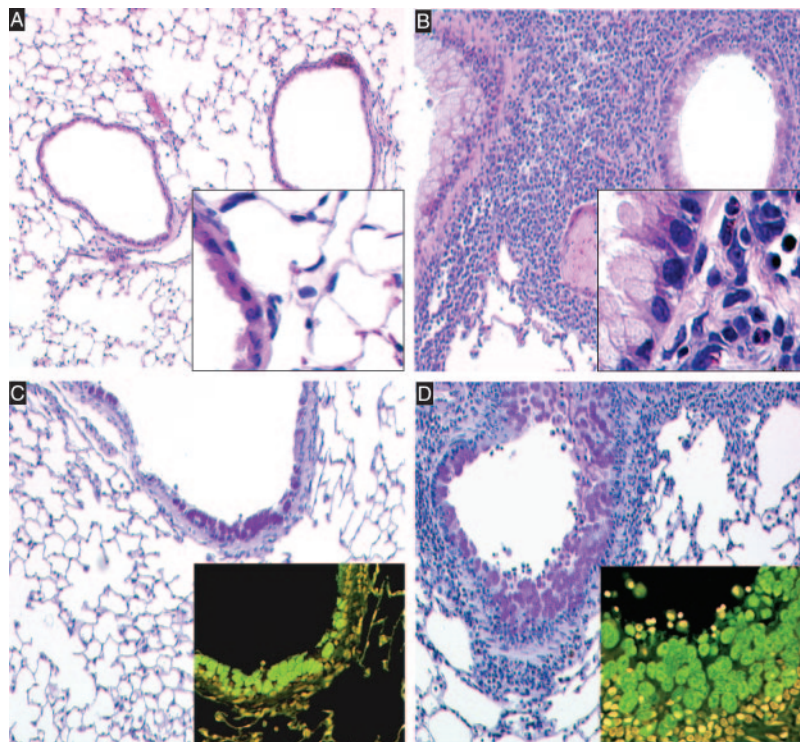
^c Serum Igs (expressed as U/ml).

^d *, $p < 0.05$ compared with naïve groups.

^e Cytokines (expressed as pg/ml) in culture supernatants.

Data are expressed as mean ± SEM; $n = 3$ –4 per group. Results are representative of three independent experiments.

FIGURE 6. Representative light photomicrographs of paraffin-embedded sections of murine lung tissue following repeated OVA aerosolization in cutaneous- or mucosally primed mice. *A* and *B*, H&E-stained sections from cutaneous- and respiratory mucosal-primed mice, respectively, 72 h following repeated OVA (5 $\times$) aerosolization. *C* and *D*, PAS-stained sections for goblet cells (magenta) from cutaneous- and respiratory mucosal-primed mice, respectively. *Insets* in *C* and *D* show PAS-stained sections viewed under color inversion to better define goblet cell hyperplasia. Original magnification of panels, $\times 100$. Magnification of *insets*, $\times 400$.



observed no significant quantitative or qualitative changes in total PB leukocytes, including no evidence of eosinopenia, following OVA aerosolization in cutaneous Ag-primed mice (Table II). Indeed, the PB leukocyte profile in these mice was statistically not different from naive controls. Of note, cutaneous Ag-primed mice, albeit unresponsive to OVA aerosolization, still exhibited clear signs of Th2 sensitization, including enhanced levels of serum OVA-IgE and IgG1, and Th2-cytokine (IL-4, IL-5, and IL-13) production by OVA-stimulated splenocytes *in vitro* (Table II).

*Histological evaluation of lung tissue and development of AHR following respiratory Ag exposure *in vivo**

We conducted a detailed histological analysis on lung sections taken from cutaneous- and respiratory mucosally primed mice following OVA aerosolization *in vivo*. In agreement with findings in BAL, analysis of lung tissue from naive mice (data not shown) and pOVA $\times 3$ -primed mice exposed to five OVA aerosols revealed similar histological features with no overt airway eosinophilic inflammation (Fig. 6A) and goblet cell hyperplasia (Fig. 6C). In contrast, OVA re-exposure to mice initially mucosally sensitized to OVA, led to marked peribronchial and perivascular inflammation that was distinctly eosinophilic and mononuclear in nature (Fig. 6B) and pronounced goblet cell hyperplasia and mucus production (Fig. 6D). To ascertain whether aerosolized OVA resulted in a decline in pulmonary physiological responses in cutaneous Ag-primed mice, we assessed airway responsiveness to i.v. administered MCh *in vivo*, 72 h following the fifth OVA exposure. As indicated in Fig. 7, repeated OVA aerosolization did not lead to AHR in cutaneous Ag-primed mice but rather produced a MCh-responsiveness curve similar to that observed in naive controls. In contrast, respiratory mucosal-primed mice experienced significant increases in RRS following OVA exposure. Altogether, these BAL, histological, and AHR data unequivocally demonstrate that cutaneous Ag-primed mice fail to mount allergic airways disease upon respiratory Ag recall.

Differential LN involvement during cutaneous or respiratory mucosal priming

Impelled by the observation that cutaneous Ag-primed mice were able to evoke Th2-immune effector responses in local cutaneous sites but not in remote mucosal compartments like the lung, we conducted a comprehensive examination of various LNs during cutaneous- and respiratory mucosal-priming regimens to better ascertain whether the site of Th2 differentiation was indicative of the site-restricted nature of the ensuing Th2-memory response. To this end, we harvested various peripheral/skin (inguinal and axillary)

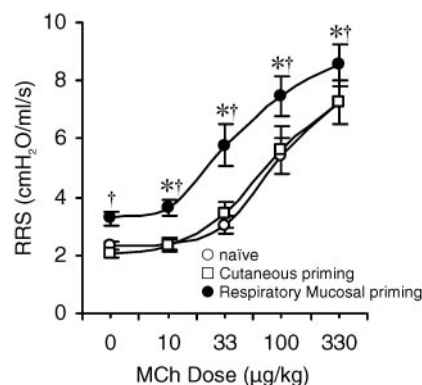


FIGURE 7. Respiratory exposure to aerosolized Ag fails to elicit AHR in cutaneous Ag-primed mice. Mice were initially sensitized to OVA via the skin or respiratory mucosa and 3–4 wk later were exposed to aerosolized OVA for 5 consecutive days. Airway responsiveness to i.v. administered MCh was assessed 72 h following the last OVA aerosolization. The graph depicts airway resistance (RRS) to increasing doses of MCh in cutaneous- and respiratory mucosal-primed mice, and in naive controls. Results are expressed as mean $\pm$ SEM; $n = 7$ –11 per group from two experiments; *, $p < 0.05$ compared with naive; †, $p < 0.05$ compared with cutaneous-primed mice.

and mucosal (mediastinal, cervical, and mesenteric) LNs at several time points during cutaneous and respiratory mucosal priming, and assayed for the presence of Ag-specific proliferation and Th2 differentiation *in vitro*. For simplicity, we report only the peak responses observed for each priming regimen. First, we observed Ag-specific proliferation in skin-draining inguinal and axillary LNs, but not lung-draining mediastinal (Fig. 8A) or cervical LNs (data not shown), following cutaneous Ag priming. This was in complete contrast to mice primed via the respiratory mucosa, which evoked Ag-specific proliferative responses solely in mediastinal, and not skin-draining, LNs (Fig. 8A). Proliferative responses were also observed in deep cervical LNs in mucosally

primed mice (data not shown). Secondly, Th2 differentiation, as indicated by archetypic Th2-cytokine (IL-4, IL-5, IL-10, and IL-13) production by OVA-stimulated LN cells *in vitro*, occurred solely in skin-draining inguinal and axillary LNs following GG-based cutaneous Ag priming, as opposed to respiratory mucosal priming which resulted in Th2-cytokine production in mediastinal, but not skin-draining, LNs (Fig. 8B). Interestingly, not only were the levels of Ag-specific proliferation and Th2-cytokine production comparable between priming regimens, but both regimens failed to mount significant Ag-specific proliferative and Th2-cytokine responses in distant mucosal LNs draining the gastrointestinal mucosa (i.e., mesenteric LNs; data not shown).

Discussion

Contact, ingestion, and inhalation of otherwise innocuous environmental allergens can trigger harmful and sometimes fatal allergic responses in a growing fraction of the population. It is of interest that while allergen exposure is pervasive enough to interact with various body surfaces (skin, lung, and gut) the clinical expression of allergic disease is largely compartmentalized. Indeed, allergic individuals rarely present with concurrent multiple-organ disease but, rather, with manifestations that privilege a particular site. Whether the initial site of allergen exposure at the time of sensitization, imprints the ensuing Th2 memory response with lasting tissue-selective tropism determining the clinical expression of allergic disease (e.g., skin allergy, food allergy, respiratory allergies), has not been formally investigated. Here, we have examined whether skin-targeted delivery of Ag could lead to Th2 sensitization, and give rise to Th2 immune-inflammatory responses upon Ag recall both in local (skin) and remote mucosal sites, such as the respiratory tract.

To test these concepts, we confined Ag delivery to the skin by way of cutaneous particle bombardment with OVA-encoding plasmids via GG technology, which has previously been shown to exclusively target the epidermal/dermal layers of skin (41–44), lead to transient but high Ag expression (42), and successfully induce both cellular and humoral immunity in several Ag systems (45–48). Our data show that cutaneous Ag priming via the GG induced a distinctive Th2-polarized humoral and cytokine profile. Moreover, and consistent with a Th2-polarized response, production of IFN- γ (a prototypic Th1-affiliated cytokine) by OVA-stimulated splenocytes or LN cells was not significantly elevated compared with controls. This Th2 bias associated with cutaneous Ag exposure is consistent with several reports demonstrating the preferential induction of Th2-associated Igs (IgE and IgG1>IgG2a) and cytokines (IL-4>IFN- γ) following GG- (30–32, 49) or epicutaneous (e.c.)-based (50–53) immunization. Likewise, recent reports have documented that GG bombardment of secreted Ag plasmids strongly biased responses toward type 2, inducing IL-4 producing CD4 and CD8 T cells (54). Importantly, we also observed robust IL-10 production following *in vitro* OVA-stimulation of splenocytes and draining LN cells, which is consistent with a recent report demonstrating a critical role for IL-10 in the development of Ag-specific Th2 skin responses in a murine model of allergic dermatitis (55). Taken together, these findings demonstrated that cutaneous Ag priming via the GG effectively induced Ag-specific Th2 immunity.

Following cognate interaction with Ag in local draining LNs, naive CD4⁺ T cells integrate costimulatory and cytokine signals, to develop into effector and memory Th2 cells. In addition to lymphocyte effector specialization, recent experimental observations have suggested that within distinct draining LNs tissue-selective homing properties are imprinted on T cells that privilege them to

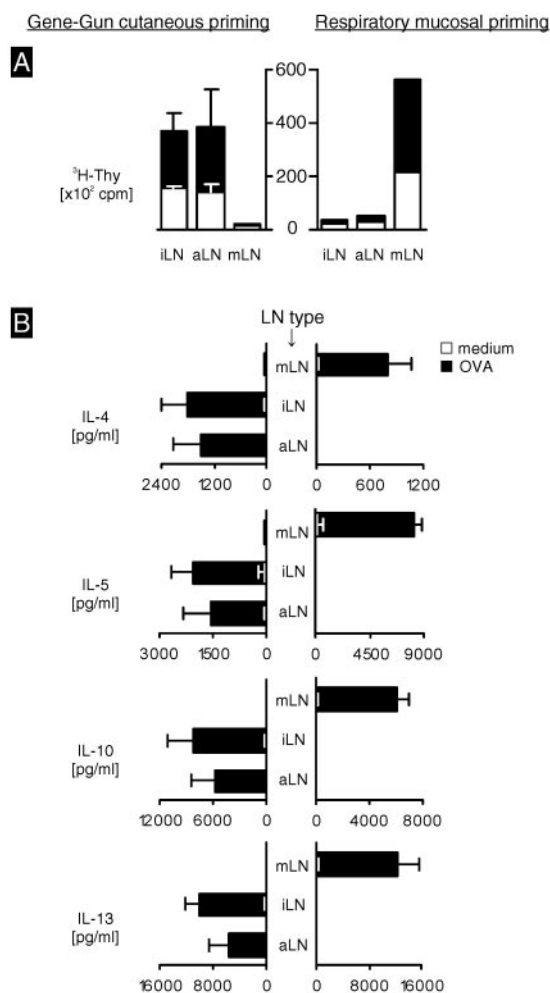


FIGURE 8. Disparate proliferative responses and Th2-cytokine production in skin/peripheral and thoracic lymph nodes following cutaneous and respiratory mucosal priming. At various time points during cutaneous or respiratory mucosal priming, individual skin (inguinal (iLN) and axillary (aLN)) and thoracic (mediastinal (mLN)) LNs were excised and examined for Ag-specific proliferative responses (A) and Th2-cytokine production (B) *in vitro*. Ag-specific proliferation (A) was assessed by [^3H]Thy incorporation following *in vitro* stimulation of harvested LN cell suspensions with OVA or medium alone for 72 h. For Th2 cytokine production (B), LN cell suspensions were stimulated by OVA or medium alone for 120 h *in vitro*, and the supernatants were measured for IL-4, -5, -10, and -13 by multicytokine Luminex assay (see *Materials and Methods*). Graphs show results for peak responses during cutaneous priming (72 h after the third GG immunization) and respiratory mucosal priming (day 9 of the protocol). Data represent mean $\pm$ SEM and representative of two independent experiments; $n = 3$ –4 per group. Data in A show pooled proliferative responses (from four mice) for the respiratory mucosal priming group.

survey sites most likely to contain their cognate Ag (8, 56). Therefore, we examined various sets of LNs during cutaneous Ag priming to assess where Th2 differentiation had occurred. We observed a pronounced expansion of skin-draining, but not gut- or lung-draining, LNs during cutaneous Ag priming, including a marked expansion of activated CD4⁺ T cells and MHCII⁺ B cells. This expansion was likely the consequence of Ag-specific activation, because proliferative responses and Th2-cytokine production were exclusively induced upon in vitro OVA stimulation. Importantly, Ag-specific proliferation and Th2-cytokine production were detectable solely in skin-draining LNs. Taken together, these data indicate that Th2 differentiation had occurred principally in skin-draining LNs during priming.

Next, we examined whether mice Th2-sensitized via the skin could mount Th2-polarized immune-inflammatory responses upon Ag recall at different sites/compartments. Indeed, Ag recall to the skin by way of i.d. ear injection of OVA protein provoked late-phase cutaneous responses, as demonstrated by an increase in ear thickness with a concomitant influx of eosinophils into the ear tissue. This was accompanied by pronounced expansion and Th2-cytokine production (IL-4, -5, and -13) in the local ear (auricle) LN draining the site of OVA injection but not the contra-lateral LN draining the site of vehicle injection (data not shown). Because OVA recall to the skin evoked Th2-cellular responses, we examined whether systemic (i.v.) OVA recall could elicit anaphylactic-like reactions in cutaneous Ag-primed mice. In fact, i.v. injection of OVA protein led to severe anaphylactic shock. Finally, we determined whether OVA recall to a remote mucosal site such as the lung could evoke Th2 immune-inflammatory responses in cutaneous Ag-primed mice. Unlike local (skin) or systemic Ag recall, repeated respiratory exposure to aerosolized OVA failed to instigate Th2 immune-inflammatory responses in the airway. Of particular importance, only mice initially primed to OVA via the respiratory mucosa (i.e., respiratory mucosal priming) mounted robust allergic airway inflammation. Given that cutaneous Ag priming led to Th2 differentiation in skin-draining LNs, we examined different LN types during respiratory mucosal priming. Close examination of different LNs demonstrated, unequivocally, Ag-specific proliferation and Th2-cytokine production solely in thoracic LNs which was in complete contrast to the LNs involved during cutaneous Ag priming. To our knowledge, these findings provide the first demonstration of compartmentalized Th2 immunity in an in vivo model of Th2 sensitization.

Previous studies in Th2 skin-sensitized mice via e.c. application of Ag through occlusive patch technology, tape-stripping, and removal of the stratum corneum, have resulted in modest airway eosinophilic inflammation following Ag inhalation (52, 53). It may be of significance to note that Ag inhalation in these studies was initiated acutely while the initial inflammatory response in the skin may not have fully resolved—thus not a memory response—and hence may have reflected mere mobilization of eosinophils from the site of e.c. exposure. This notion is consistent with the lack of significant lymphocyte infiltration into the lung (52, 53) and expansion of locally draining (thoracic) LNs (50), which would be expected to accompany an immune-inflammatory response that is induced de novo in the lung. The apparent discrepancy between these and our findings may also reflect the rigorous site-restricted Ag delivery modality of the GG system. For instance, it is uncertain whether e.c. Ag-delivery confines Ag exclusively to the skin, or results in leakage into the systemic circulation or distant tissue microenvironments (57), mimicking that of other transdermal drug delivery systems. The latter possibility is likely because the full-scale AD-like lesions observed in some mice (51, 52) may accelerate skin barrier disruption and further increase the degree of Ag

penetration. Hence, the precise site of Ag presentation is ambiguous and may, in fact, involve multiple secondary lymphoid organs, thus imprinting diverse homing potentials. Indeed, transcutaneous immunization methods, also via disruption of the stratum corneum and occlusive patch technology, have been recently shown to result in the trafficking of Ag-primed APCs from the skin to distant Peyer's patches resulting in the generation of strong mucosal Ab and CTL responses (58). Conversely, studies using skin-restricted GG-based immunization strategies have demonstrated that, while offering protection against systemic or skin viral challenges, skin-restricted immunization strategies show limited therapeutic efficacy in ablating viruses at mucosal sites (59–61). In fact, only when GG-based vaccine strategies target mucosal surfaces, such as the anorectal epithelium or oral mucosa, is protection against mucosal pathogens secured (60–63).

Compartmentalization of immune responses likely results from specific homing patterns of memory and effector T lymphocytes which are largely defined by unique chemokine receptor profiles (1). Recent investigation has identified, in humans, a population of skin-homing CD4⁺ T cells expressing the cutaneous lymphocyte-associated Ag (2, 11, 55, 64–66) and the chemokine receptors CCR4 and CCR10. Moreover, these cells have been demonstrated to migrate preferentially to inflamed skin via tissue-specific chemokine gradients, including CCL17 (TARC; Ref. 67) and CCL27 (CTACK; Refs. 15 and 17). The involvement of this lymphocyte subset in our system has yet to be proven, due in part by the unavailability of Abs definitively phenotyping the murine equivalent of the skin-homing cutaneous lymphocyte-associated Ag on murine T cells. However, it is likely that Th2 compartmentalization following skin-targeted sensitization may be maintained by a population of skin homing T cells that lack the appropriate homing molecules to access the respiratory tract, and are therefore prevented from initiating an airway inflammatory response upon Ag inhalation.

In summary, we have demonstrated that Ag DNA delivery selectively to the skin via GG elicits a Th2 immune response whose effector activity remains locally confined to the skin sparing mucosal sites, such as the respiratory mucosa. In our view, these findings have several distinct but related implications. First, they suggest that cutaneous allergen contact is unlikely to directly lead to allergic airway diseases if the Ag, during sensitization, remains compartmentalized within the skin. Second, they emphasize the potential advantages of GG-mediated skin immunization as a strategy to generate immunomodulatory responses for skin diseases. Although we have documented a Th2 profile in this study, code-livery of other cytokines may be able to generate distinct immunoregulatory responses. Importantly, the site-restriction of the effector response allays fears of launching an inflammatory response at a site (i.e., mucosae) where it is not needed and can, in fact, be detrimental.

Acknowledgments

We extend our thanks to Dr. Mark D. Inman and Jennifer Wattie for valuable discussion and expert technical assistance, and Stacey A. Ritz, Mahmoud Pouladi, Beata U. Gajewska, and Ryan E. Wiley for helpful discussion and critique. We also gratefully acknowledge the secretarial assistance of Mary Kiriakopoulos.

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