



## **IL-4-Dependent Th2 Collateral Priming to Inhaled Antigens Independent of Toll-Like Receptor 4 and Myeloid Differentiation Factor 88**

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# IL-4-Dependent Th2 Collateral Priming to Inhaled Antigens Independent of Toll-Like Receptor 4 and Myeloid Differentiation Factor 88<sup>1</sup>

Stephanie C. Eisenbarth,<sup>2\*</sup> Alex Zhadkevich,\* Patricia Ranney,\* Christina A. Herrick,\*<sup>†</sup> and Kim Bottomly\*<sup>†</sup>

Allergic asthma is an inflammatory lung disease thought to be initiated and directed by type 2 helper T cells responding to environmental Ags. The mechanisms by which allergens induce Th2-adaptive immune responses are not well understood, although it is now clear that innate immune signals are required to promote DC activation and Th2 sensitization to inhaled proteins. However, the effect of ongoing Th2 inflammation, as seen in chronic asthma, on naive lymphocyte activation has not been explored. It has been noted that patients with atopic disorders demonstrate an increased risk of developing sensitivities to new allergens. This suggests that signals from an adaptive immune response may facilitate sensitization to new Ags. We used a Th2-adoptive transfer murine model of asthma to identify a novel mechanism, termed "collateral priming," in which naive CD4<sup>+</sup> T cells are activated by adaptive rather than innate immune signals. Th2 priming to newly encountered Ags was dependent on the production of IL-4 by the transferred Th2 population but was independent of Toll-like receptor 4 signaling and the myeloid differentiation factor 88 Toll-like receptor signaling pathway. These results identify a novel mechanism of T cell priming in which an Ag-specific adaptive immune response initiates distinct Ag-specific T cell responses in the absence of classical innate immune system triggering signals. *The Journal of Immunology*, 2004, 172: 4527–4534.

Activation of naive CD4<sup>+</sup> T cells requires the delivery of two signals by the APC: MHC class II-presented Ag and costimulation from B7 or other related costimulatory molecules (1). Control of these signals on the APC comes from the triggering of innate immune system receptors (e.g., Toll-like receptors (TLR)<sup>3</sup>) by microbial molecular patterns (2, 3). Subsequent to or during priming, helper T cells differentiate into either Th1 or Th2 cells, as defined by the pattern of cytokine production (4). Although a number of signals have been identified that are capable of influencing Th cell differentiation, cytokine signaling plays a dominant role (IL-12 in the case of Th1 cells and IL-4 in the case of Th2) (5). What remains unclear is what signals govern the initial priming of helper T cells to non-pathogen-associated environmental protein Ags, as occurs in disease states such as asthma.

Emerging evidence from murine models of asthma and epidemiological studies indicate that, like Th1 immunity, the priming of naive T cells into Th2 cells to protein Ags also requires activation of the innate immune system (6–10). Numerous studies have

shown that TLR activation and signaling through the common TLR adaptor protein myeloid differentiation factor 88 (MyD88) result in dendritic cell activation, a necessary step in the priming of naive T cells. We and others have further shown that induction of Th2 airway responses to one protein Ag, OVA, requires activation of the innate immune system through LPS stimulation of TLR4 (7, 8). A fundamental question, however, is whether Th2 sensitization to protein Ags always requires activation of the innate immune system or whether innate immune system-independent mechanisms of T cell priming exist.

Clinical evidence suggests that once sensitized to one allergen, atopic patients are more likely to become sensitized to other environmental allergens (11–13). This implies that the threshold for Th2 sensitization is lower in patients with chronic allergic inflammation. It also suggests that Th2 sensitization to Ags during ongoing inflammation may not follow the same rules governing priming of naive T cells. Therefore, we sought to determine whether an ongoing Th2 adaptive immune response in the lung would influence T cell priming to subsequent inhaled Ags and could perhaps bypass innate immune system signal requirements.

We observed that activated Th2 cells (either adoptively transferred or primed *in vivo*) facilitated subsequent priming of endogenous naive T cells to new Ags in what we have termed collateral priming. Unlike the situation in a naive host, we found that collateral priming to an inhaled protein Ag during an active Th2 response did not require TLR4 or MyD88 signaling. Instead, this priming was dependent on IL-4 production by the activated Th2 population. Although IL-4 is known to direct Th2 differentiation, this work suggests a potential role for IL-4 in the initial activation of naive T cells (priming) in that IL-4 may substitute for TLR adjuvant-type initiation signals. Because TLR ligands control the initiation of adaptive immune responses by influencing the APC activation state, we propose an analogous model of APC activation

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<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; TLR4d, TLR4 defective; BAL, bronchoalveolar lavage; LN, lymph node; DLN, draining LN; WT, wild type; KLH, keyhole limpet hemocyanin; KO, knockout.

by noninfectious stimuli such as cytokines, resulting in the amplification of other adaptive immune responses. These findings may also explain the clinical observation that sensitization to one allergen is a risk factor for developing subsequent Th2 responses and that Th2 priming during active inflammatory processes can occur independently of innate immune system signaling.

## Materials and Methods

### Animals

BALB/cJ (wild type (WT)) and C.C3H-*Tlr4*<sup>Lps-d</sup> (TLR4 defective (TLR4d)) mice on a BALB/c background (see Fig. 5) were purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88-deficient mice were generated as previously described (14) and kindly provided by R. Medzhitov on a BALB/c background (Yale University, New Haven, CT). BALB/cAnNCr mice (WT in all other figures) were purchased from the National Cancer Institute (Frederick, MD). TCR-transgenic CD4 T cell donors were either DO11.10 mice backcrossed onto a BALB/c  $\alpha\beta^{-/-}$  background or IL-4<sup>-/-</sup> DO11.10 mice that were bred in our facility (15). Six- to 10-wk-old female mice were used in all experiments.

### Generation of Th2 cells and adoptive transfer

CD4<sup>+</sup> T cells were isolated from the spleens of BALB/c or transgenic mice by negative selection using Abs to MHC class II I-A<sup>b</sup> (212.A1), CD8 (TIB 210), B220 (TIB 164), and FcR (24G2) followed by anti-Ig-coated magnetic beads (Polysciences, Warrington, PA). Syngeneic T-depleted splenocytes were used as APCs and were prepared by Ab-mediated rabbit complement lysis using Abs to CD4 (GK1.5), CD8 (TIB 105), and Thy-1 (Y19) followed by mitomycin C treatment (Sigma-Aldrich, St. Louis, MO).

To generate Th2 cells,  $0.5 \times 10^6$ /ml CD4<sup>+</sup> T cells and  $1.0 \times 10^6$ /ml APCs were cultured with 5 mg/ml pOVA<sub>323-339</sub>, 10 U/ml recombinant murine IL-2 (Roche), 10 ng/ml recombinant murine IL-4 (PeproTech, Rocky Hill, NJ), and anti-IFN- $\gamma$  (XMG1.2). After 4 days,  $5 \times 10^6$  cells were injected i.v. into BALB/c mice. DO11.10 Th2 cell purity before injection ranged from 92 to 98% CD4<sup>+</sup>KJ1-26<sup>+</sup> by FACS analysis (see below). An aliquot of Th2 cells was retained for in vitro restimulation and analysis by ELISA (see below).

### Flow cytometry analysis

Transgenic T cells were analyzed by FACS for purity before adoptive transfer and cells harvested from the bronchoalveolar lavage (BAL) were analyzed for the presence of DO11.10 transgenic T cells by CyChrome anti-CD4 (L3T4; BD PharMingen, San Diego, CA) and biotinylated anti-clonotypic Ab (KJ1-26) staining (16) followed by FITC-avidin D (Vector Laboratories, Burlingame, CA). The CD4<sup>+</sup>KJ1-26<sup>+</sup> cells in the BAL during the secondary challenge (see Fig. 2B) were H57<sup>+</sup> (a pan-TCR  $\beta$ -chain Ab; BD PharMingen), indicating that the absence of KJ1-26 staining was not simply due to TCR down-regulation.

### Airway challenge

For the primary challenge, mice were exposed 24 h after Th2 adoptive transfer to either 5  $\mu$ g of OVA (grade V; Sigma-Aldrich) or 5  $\mu$ g of OVA and 5  $\mu$ g of BSA (fraction V; Invitrogen, Grand Island, NY) intranasally on days 0 and 1. Secondary challenge was performed 18 days later with either 5  $\mu$ g of OVA or BSA on days 18 and 19. Mice were sacrificed on day 22 (4 days after the airway Ag exposure). In Fig. 3D, keyhole limpet hemocyanin (KLH; Sigma-Aldrich) replaced BSA in the collateral priming protocol.

### Inhalational and i.p. priming and challenge protocol

For Fig. 5A mice (BALB/c and TLR4d) were sensitized intranasally with 100  $\mu$ g BSA in 50  $\mu$ l of PBS on days 0, 1, and 2 as previously described (8). Mice were then challenged intranasally on days 14, 15, 18, and 19 with 25  $\mu$ g of BSA and sacrificed on day 21 for BAL analysis. In Fig. 3E, BALB/c mice were sensitized to OVA via i.p. injection of 100  $\mu$ g of OVA in 2 mg of aluminum hydroxide (Pierce, Rockford, IL) in a total volume of 0.25 ml. Ten days later, mice were exposed to either 25  $\mu$ g of BSA alone or 25  $\mu$ g of OVA and 25  $\mu$ g of BSA together (Ag doses were altered in this experiment to account for the difference in original Th2 priming conditions.) To test for a BSA-specific T cell response, mice were challenged 2 wk later with 25  $\mu$ g of BSA and BAL analysis was done. No Th2 cell transfer was done in either of these experiments.

### Analysis of BAL

BAL inflammatory cells were obtained by cannulation of the trachea and lavage of the airway lumen with PBS as previously described (17). Cytospin slides were prepared by H&E staining with Diff-Quick (Dade Behring, Newark, DE).

### Determination of statistical significance

Statistical significance was determined using the Mann-Whitney *U* test of either total BAL numbers or absolute BAL eosinophil numbers. A *p* < 0.05 was considered to be significant.

### Lung histology

Paraffin-embedded coronal lung sections were prepared as previously described (18) and stained with H&E or periodic acid-Schiff.

### Determination of serum Ab concentration

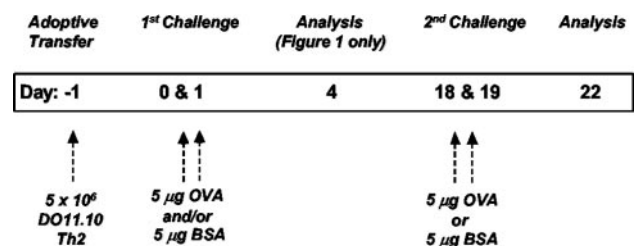
We obtained serum on day 22 for measurement of BSA-specific IgG1 and IgE Abs by ELISA as previously described (18). Briefly, plates were coated with 50  $\mu$ g/ml BSA in 0.1 M NaHCO<sub>3</sub> at 4°C overnight. A 1% milk solution was used to block plates. Sera were incubated for 1 h followed by biotin-labeled rat anti-mouse IgG1 (BioSource International, Camarillo, CA) or biotin-labeled rat anti-mouse IgE (LO-ME-3; BioSource International). Streptavidin-conjugated HRP (Zymed Laboratories, San Francisco, CA) was added at 37°C, followed by tetramethylbenzidine substrate (Bio-Tek, Winooski, VT). Hyperimmune serum from BSA/alum-immunized BALB/c mice was used for a standard and set at 100 U/ml. Total IgE was measured as previously described (18) using an anti-IgE coating Ab from the Binding Site (Birmingham, U.K.) and the IgE-3 standard from BD PharMingen.

### Lymph node (LN) cytokine production

Mediastinal LNs were harvested and pooled from each group at time of sacrifice. Cells were isolated and stimulated in vitro with 200  $\mu$ g/ml OVA or BSA and syngeneic T cell-depleted splenocytes. We measured cytokines in culture supernatants using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Levels of detection were 25.0 pg/ml (IL-4), 125.0 pg/ml (IL-5), 31.2 pg/ml (IL-13), and 1.9 ng/ml (IFN- $\gamma$ ).

### Magnetic cell sorting

Transgenic T cells were separated from the draining LN (DLN) using MACS according to manufacturer's specifications. Cells were separated using KJ1-26 and superparamagnetic MicroBeads conjugated to streptavidin (Miltenyi Biotec, Auburn, CA). Confirmation of separation was assessed by FACS analysis.



**FIGURE 1.** Murine asthma model protocol. All experiments except those represented in Figs. 5A and 3E follow the above-diagrammed timeline for DO11.10-transgenic Th2 transfer and airway Ag challenge regimen. In Fig. 2, airway responses after both the first Ag challenge (days 0 and 1) and the second Ag challenge (days 18 and 19) are included. The inflammatory response after the first challenge resolves by day 18 and therefore is the point at which we deliver the second challenge. All figures subsequent to Fig. 2 show airway responses only after the second challenge (days 18 and 19). For these remaining figures, the Ags administered during both the first and second challenges are indicated within the figure. It should be emphasized that all BAL analyses were done 3 days after the last airway Ag exposure (day 4 or 22 for either the first or second challenge, respectively).

**Results**

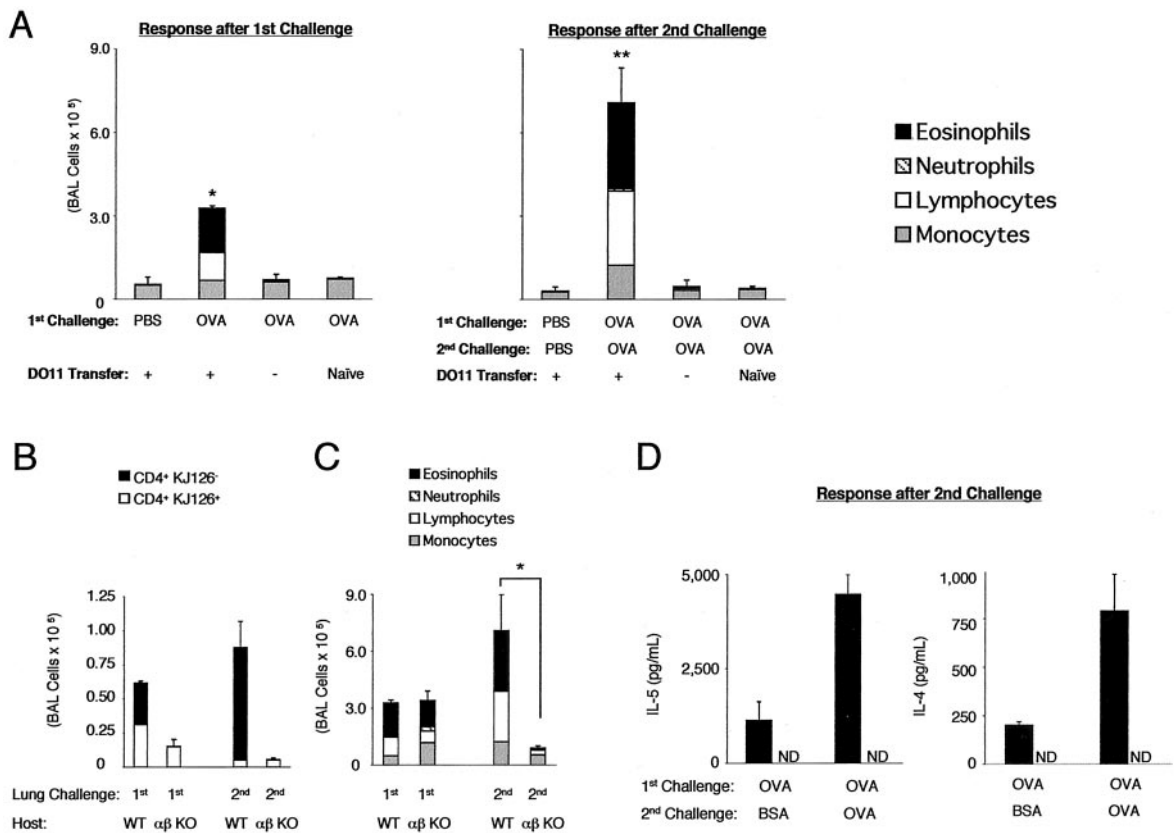
*Long-lived sensitization to OVA in an adoptive transfer model of asthma is dependent on endogenous, Ag-specific CD4<sup>+</sup> T cells*

To investigate how an active Th2 allergic response influences sensitization to other allergens, we used a murine asthma model involving the adoptive transfer of in vitro-differentiated DO11.10 Th2 cells expressing a transgenic TCR specific for the 323–339 peptide of OVA (Fig. 1). As previously reported, DO11.10 Th2 cell transfer into naive BALB/c mice and exposure to inhaled OVA results in local Th2 cell activation, eosinophilic lung inflammation and mucus production (19). It has been noted, however, that transferred transgenic CD4<sup>+</sup> T cells do not survive in vivo after primary Ag challenge in the absence of an adjuvant (20, 21). Therefore, to establish a model of ongoing Th2 inflammation, we first asked whether an immunocompetent host could maintain Th2-driven responses to multiple OVA challenges after transfer of a TCR-transgenic Th2 population.

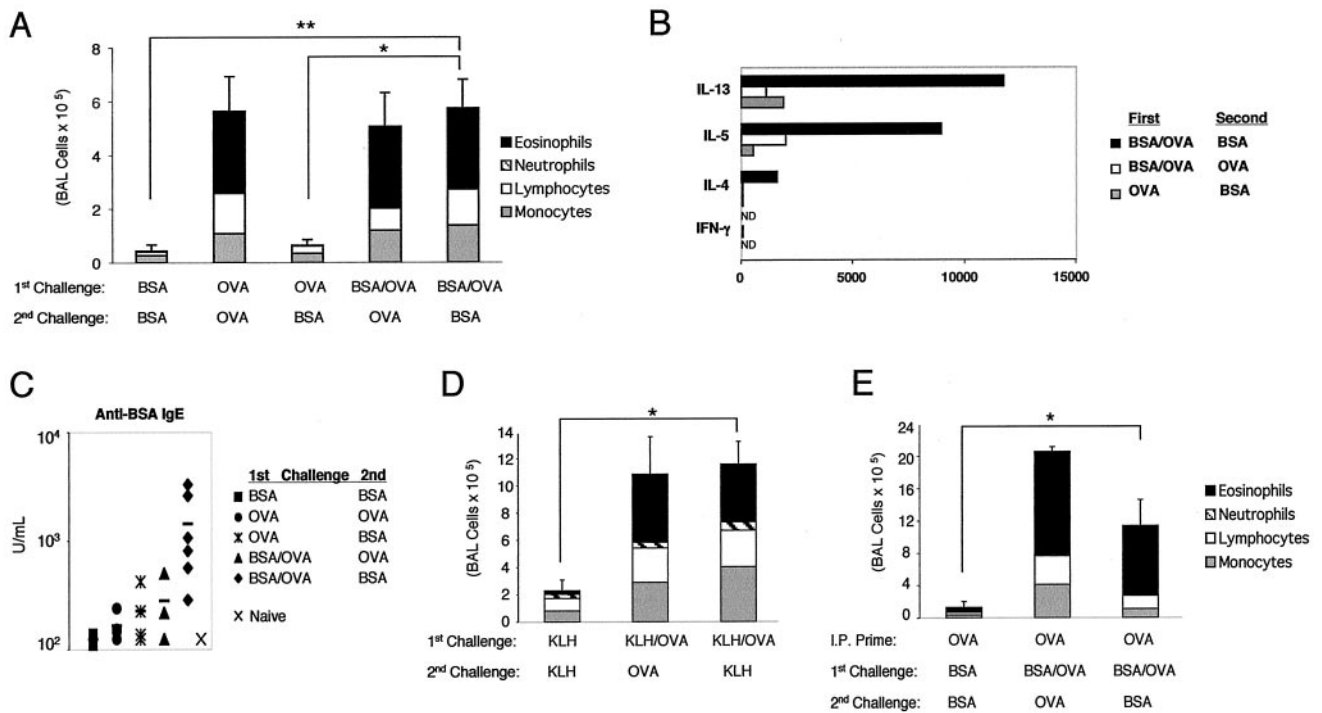
Consistent with previous studies, DO11.10 Th2 transfer and challenge with OVA results in airway eosinophilic inflammation (Fig. 2A, first panel). Subsequent OVA challenge results in augmented BAL (Fig. 2A, second panel) and lung (data not shown) eosinophilic influx. These characteristic lung changes were both Ag and Th2 transfer-dependent as lung inflammation was not ob-

served after either PBS challenge following transfer of Th2 cells or OVA challenge in the absence of Th2 transfer (Fig. 2A). Similarly, the transfer of naive transgenic T cells followed by OVA challenge failed to induce inflammation above baseline (Fig. 2A).

Transferred DO11.10 Th2 cells were recruited into the airways after inhaled OVA challenge, as demonstrated by staining with the TCR clonotypic Ab KJ126 (22). However, consistent with previous reports, the proportion and number of KJ126<sup>+</sup> T cells in the BAL was reduced by >90% following the second challenge (Fig. 2B, WT) (20, 21). Lung DLN, blood, and spleen showed a similar decrease of KJ126<sup>+</sup> cell number (data not shown). Interestingly, this loss of KJ126<sup>+</sup>CD4<sup>+</sup> T cells did not lead to a corresponding loss of pulmonary inflammation, as the total number of inflammatory cells and eosinophils in the BAL were augmented after the second challenge (Fig. 2, A and C, WT). Instead, there was a 3-fold increase in CD4<sup>+</sup>KJ126<sup>-</sup> endogenous T cells after the secondary challenge (Fig. 2B, WT). This expansion of endogenous T cells did not occur in the absence of transferred DO11.10 Th2 cells (data not shown). Taken together, these data suggest that an endogenously derived CD4<sup>+</sup> T cell population developed and was dependent on the presence and activation of the transferred DO11.10 Th2 cells, and that this new Th2 population maintained the eosinophilic pulmonary response subsequent to the primary Ag challenge.



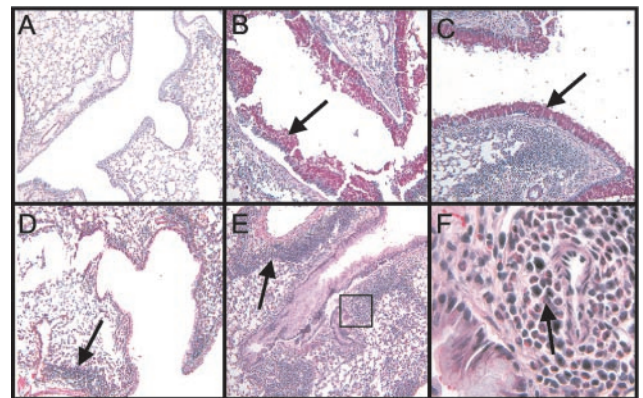
**FIGURE 2.** Airway inflammation induced by adoptive transfer of transgenic Th2 cells is maintained after secondary challenge by an endogenous, Ag-specific T cell. *A*, BAL cell differential 4 days after first or second inhalational exposure to either PBS or OVA is represented by stacked bars. Mice received DO11.10 Th2 cells (+), no cells (-), or naive DO11.10 CD4<sup>+</sup> T cells (Naive) before first inhalational challenge. Error bars are based on total BAL cell numbers. \*, *p* < 0.05 (vs all other groups following first challenge); \*\*, *p* < 0.03 (vs all other groups following second challenge). *n* = 4 mice/group. *B*, Absolute number of CD4<sup>+</sup>KJ126<sup>+</sup> vs CD4<sup>+</sup>KJ126<sup>-</sup> cells from live gate of BAL in BALB/c (WT) or αβ<sup>-/-</sup> (KO) mice 4 days after the first or second OVA exposure. The transferred transgenic cell number in the BAL is represented by open bars and is reduced by 90% in WT mice (*p* < 0.05). One representative experiment of three is shown. *C*, BAL cell differential from the above groups represented as stacked bar graph. Error bars based on total BAL cell numbers. \*, *p* < 0.02. *n* = 4 mice/group. *D*, IL-5 and IL-4 production by cells pooled from the DLN of indicated groups stimulated in vitro with APCs and either 200 μg/ml OVA (■) or 200 μg/ml BSA (□) (see Fig. 3A for BAL responses given this Ag challenge protocol). LN cells were depleted of residual KJ126<sup>+</sup> cells by MACS bead separation before simulation. IFN-γ was not detectable.



**FIGURE 3.** An activated Th2 population contributes to the priming of a Th2 response to a distinct Ag. **A**, BAL cell differential at day 22 (4 days following second challenge) from BALB/c mice injected with DO11.10 Th2 cells and challenged with 5  $\mu$ g of indicated Ag(s) during the primary and secondary airway exposure. Error bars are based on total BAL cell numbers. \*,  $p < 0.002$ ; \*\*,  $p < 0.01$ ,  $n = 4-6$  mice/group. **B**, IL-4 (picograms per milliliter), IL-5 (picograms per milliliter), IL-13 (picograms per milliliter), and IFN- $\gamma$  (nanograms per milliliter) production by pooled DLN cells from indicated groups stimulated in vitro with APCs and 200  $\mu$ g/ml BSA. Groups exposed only to BSA during both the first and second challenge did not produce detectable Th2 cytokines ex vivo to BSA. KJ126 MACS separation did not affect the LN cytokine response to BSA. One representative experiment of four is shown. **C**, BSA-specific IgE in the sera of mice exposed to the Ag challenge protocol from **A**. Serum from unmanipulated BALB/c mice was used as a naive control. **D**, BAL response in BALB/c mice exposed to the collateral priming protocol as in **A**, except KLH was used instead of BSA. \*,  $p < 0.05$ ,  $n = 4$  mice/group. **E**, BAL response in BALB/c mice primed in vivo to OVA by i.p. injection with OVA in aluminum hydroxide on day 0. On days 10–12, mice were exposed to either 25  $\mu$ g of BSA or 25  $\mu$ g of OVA and 25  $\mu$ g BSA together to mimic the first challenge in the collateral priming protocol (**A**). Subsequent Ag challenge with 25  $\mu$ g of either OVA or BSA was done on days 29, 30, 33, and 34, and BAL analysis was done on day 36. This protocol incorporates aspects of both the collateral priming regimen from **A** and murine asthma models initiated by i.p. Ag sensitization. \*,  $p < 0.05$ ,  $n = 4$  mice/group.

To confirm the role of endogenous CD4<sup>+</sup> T cells in the maintenance of the inflammatory response, we used host mice deficient of endogenous T cells ( $\alpha\beta^{-/-}$ ). As expected, adoptively transferred Th2 cells induced similar pulmonary responses after primary challenge with OVA in WT and knockout (KO) mice. However, WT mice exhibited continued eosinophilic inflammation after two rounds of Ag challenge, whereas CD4<sup>+</sup> T cell-deficient mice could not sustain inflammation past the first OVA challenge (Fig. 2, **B** and **C**). Therefore, the endogenous CD4<sup>+</sup> KJ126<sup>-</sup> population, which expands during the secondary challenge in WT hosts, is necessary for the continued pulmonary response.

Next, we tested the Ag specificity of this activated endogenous population in our system by evaluating the response to a second Ag, BSA. In contrast to WT mice exposed to OVA during both the primary and secondary challenge, WT mice exposed to BSA during the secondary challenge did not demonstrate eosinophilic BAL or lung inflammation (see Figs. 3A and 4A). Furthermore, when lung DLN cells from these two groups were stimulated ex vivo with either OVA or BSA, only OVA was capable of stimulating Th2 cytokine production (Fig. 2D). Residual KJ126<sup>+</sup> cells were removed by MACS separation before restimulation in Fig. 2D, confirming that endogenous cells (KJ126<sup>-</sup>) were responsible for OVA-specific cytokine production. Therefore, the newly primed endogenous CD4<sup>+</sup> T cells, which were present in the lung during the secondary response and were associated with continued eosinophilic inflammation, were OVA specific.



**FIGURE 4.** Pulmonary BSA-specific responses are induced by activation of an OVA-specific Th2 population. Representative lung histology demonstrating either airway mucus secretion (periodic acid-Schiff; **A-C**) or tissue inflammation (H&E; **D-F**) following the second challenge. All mice received DO11.10 Th2 cells and were then exposed to the following Ags during the first and second challenge, respectively: **A**, OVA  $\rightarrow$  BSA (magnification,  $\times 100$ ); **B** and **D**, BSA/OVA  $\rightarrow$  OVA (magnification,  $\times 100$ ); **C** and **E**, BSA/OVA  $\rightarrow$  BSA (magnification,  $\times 100$ ); and **F**, Magnification of box in **E** showing eosinophilia ( $\times 400$ ). Arrows indicate either positive mucus staining (**B** and **C**) or eosinophilic infiltrates (**D-F**).

### Ongoing Th2 responses can induce Ag-specific CD4<sup>+</sup> T cell responses to a new Ag

The above data suggest that the initial Th2 response in the lung, responsible for initiating an endogenous CD4<sup>+</sup> OVA-specific response, produced factors capable of facilitating naive T cell priming to OVA. These results led us to question whether the original Th2 population might lower the threshold for sensitization to new allergens if present during the primary Th2 response. We tested this hypothesis by introducing an Ag that does not activate DO11.10 Th2 cells (BSA) during the initial challenge.

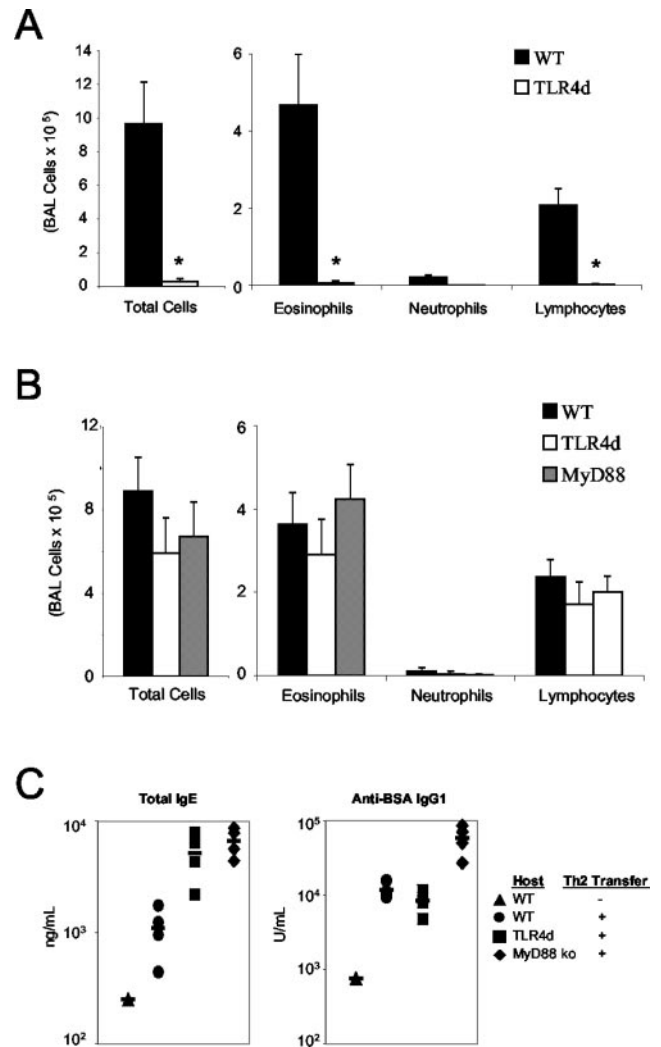
As seen in Figs. 3A and 4, B–F, if BSA and OVA were present during the first challenge, then BAL and lung tissue eosinophilia, as well as airway mucus secretion, were observed after a second challenge of either BSA or OVA alone. As expected, this correlated with the expansion of endogenously derived KJ126<sup>+</sup> CD4<sup>+</sup> T cells ( $9.06 \pm 0.76 \times 10^6$  in the DLN of mice exposed first to OVA/BSA and second to BSA vs  $1.12 \pm 0.07 \times 10^6$  in mice exposed to only BSA during both challenges). DLN cells from mice exposed to inhaled OVA/BSA and then BSA during the secondary challenge responded *ex vivo* to BSA by producing Th2 cytokines (Fig. 3B). In vivo, airway responses to BSA correlated with BSA-specific IgE (Fig. 3C) and IgG1 (see Fig. 5C, WT) production, confirming the generation of a Th2 BSA-specific response.

The priming of BSA-specific CD4<sup>+</sup> T cells depended on the activation of the transferred Th2 cells, as demonstrated by mice exposed to BSA but not OVA during the first challenge. These mice failed to elicit a pulmonary response following the second challenge of BSA (Fig. 3A), indicating that the low dose of BSA during exposure was not sufficient to invoke T cell priming but required activation of the transferred Th2 cells by OVA inhalation to generate BSA-specific responses. Conversely, if BSA was absent during the first OVA challenge of the transferred cells, subsequent BSA challenge did not result in airway (Figs. 3A and 4A) or DLN cytokine responses (Fig. 3B). To rule out that antigenic similarity between OVA and BSA accounted for the ability to prime in this model, we extended the above studies using another Ag, KLH, and found identical results (Fig. 3D). Furthermore, to rule out that collateral priming occurs only in the presence of a transgenic Th2 population, we in vivo-primed BALB/c mice to OVA (via i.p. injection of 100  $\mu$ g OVA with 2 mg aluminum hydroxide) and then exposed these mice to OVA and BSA together during the first challenge and to BSA or OVA alone during the second challenge. As before, lung eosinophilia and LN Th2 cytokine production could be elicited with BSA alone, indicating that collateral priming to BSA can be initiated by in vivo OVA-primed T cells (Fig. 3E).

These data indicate that the priming of an endogenous CD4<sup>+</sup> T cell population to a new Ag can be facilitated by the presence and activation of Th2 cells specific for a different Ag, a process we have called collateral priming. As priming under these conditions depended on an activated adaptive cellular response, we next tested whether innate immune signals, which normally govern whether T cell priming or anergy results following Ag exposure, were required.

### Collateral priming occurs normally in TLR4d and MyD88-deficient mice

We have shown previously that TLR signaling, specifically TLR4, is required for Th2 sensitization to high doses of inhaled proteins, such as OVA (8) and BSA (Fig. 5A), in naive mice. These protein Ags contain a low level of LPS that is necessary for the activation of APCs and efficient T cell priming (8). Therefore, we next eval-



**FIGURE 5.** Collateral priming is not dependent on TLR4 or MyD88 signaling. *A*, BAL eosinophils, neutrophils, and lymphocytes from WT (BALB/cJ) or TLR4d mice after intranasal sensitization to 100  $\mu$ g of BSA on days 0, 1, and 2 followed by intranasal challenge with 25  $\mu$ g of BSA on days 14, 15, 18, and 19. In the absence of Th2 transfer, priming to inhaled BSA requires intact TLR4 signaling as previously reported for the Ag OVA (7, 8). \*,  $p < 0.03$ .  $n = 6$  mice/group. *B*, BAL eosinophils, neutrophils, and lymphocytes from WT (BALB/cJ), TLR4d, or MyD88 KO mice after injection with DO11.10 Th2 cells followed by a first challenge with 5  $\mu$ g of BSA/OVA each and a second challenge with 5  $\mu$ g of BSA (see Fig. 1 for protocol). Cell numbers in WT vs TLR4d or MyD88<sup>-/-</sup> mice were not statistically different.  $n = 4$  mice/group. *C*, Serum BSA-specific IgG1 and total IgE on day 22 from WT, TLR4d, or MyD88<sup>-/-</sup> mice injected with (+) DO11.10 Th2 cells followed by primary challenge with OVA/BSA and secondary challenge with BSA. WT mice without (-) DO11.10 Th2 injection failed to mount an IgE or IgG1 response following the above challenge protocol.

uated whether TLR4 signaling and LPS play a similar role in collateral priming. DO11.10 Th2 cells were adoptively transferred into either WT (BALB/c) or C3H-Tlr4<sup>Lps-d</sup> (TLR4d, BALB/c background) mice, which have deficient signaling through TLR4. These mice were then challenged with inhaled OVA and BSA to induce the priming of endogenous CD4<sup>+</sup> T cells specific for BSA. Surprisingly, the airway eosinophilic inflammation and systemic BSA-specific IgG1 production induced by exposure to BSA/OVA followed by exposure to BSA alone was equivalent in WT and TLR4d mice, indicating that generation of endogenous BSA-specific Th2 cells is intact in the absence of TLR4 signaling (Fig. 5,

B and C). To rule out that other pathogen-associated molecular patterns may be involved in collateral priming, we repeated these experiments in MyD88-deficient animals that lack the common TLR adaptor protein necessary for efficient and complete signal transduction for the majority of described TLR ligands (23–28). Again, collateral priming to BSA was intact in these mice as evidenced by unaltered BAL eosinophilia and serum Ab responses when compared with background-matched WT mice (Fig. 5, B and C). These data suggest that the activated Th2 population itself produces factor(s) that facilitate Th2 priming to a novel Ag, which in this model, abrogated the need for TLR stimulation.

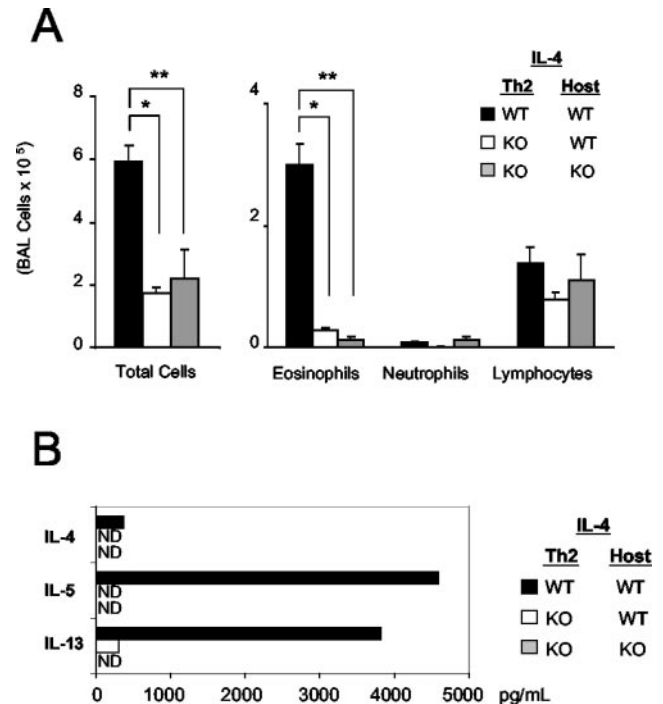
#### *IL-4 from the transferred and activated Th2 population instigates collateral priming*

We next explored what factor was responsible for enabling collateral Th2 sensitization. IL-4 is known to be essential in Th2 differentiation; however, it is not clear what role it may play in the activation of naive T cells. From *in vitro* studies, it was shown that IL-2, rather than IL-4, is required during the priming process of Th2 cells (29); subsequently, IL-4 directs differentiation (29, 30). To test the role of IL-4 in collateral priming, we transferred IL-4-deficient DO11.10 Th2 cells into WT hosts. Importantly, Th2 cells from WT and IL-4<sup>-/-</sup> DO11.10 donors produced equivalent levels of IL-5 and IL-13 (15,000 vs 22,000 pg/ml IL-5 and 21,500 vs 18,000 pg/ml IL-13, respectively). From previous work, we also know that IL-4-deficient transgenic Th2 cells can be recruited to the lung after primary Ag inhalation and are capable of inducing airway hyperresponsiveness, mucus secretion, and pulmonary inflammation (31). Therefore, if secretion of IL-4 by activated DO11.10 Th2 cells is responsible for the priming of BSA-specific cells, then eliminating IL-4 production from the transferred population should abrogate BSA-specific immunity after the secondary challenge even in an IL-4<sup>+/+</sup> host.

Fig. 6A depicts the airway inflammatory response in mice exposed to BSA and OVA during the first challenge followed by BSA during the second challenge. Whereas WT Th2 cells transferred into WT hosts resulted in the initiation of a BSA-specific Th2 response, the transfer of IL-4-deficient DO11.10 Th2 cells did not lead to priming of BSA-specific Th2 cells in either WT or IL-4-deficient hosts, as measured by BAL cell eosinophilia (Fig. 6A) and DLN Th2 cytokine production (Fig. 6B). It is important to note that the endogenous T cells in the WT host, which must eventually become BSA specific to mediate a pulmonary response to BSA challenge, are capable of producing all Th2 cytokines and therefore, if activated to BSA, should be able to generate pulmonary eosinophilic inflammation or LN Th2 cytokine responses to BSA challenge. In addition, the 3-fold reduction in total airway inflammation and the absence of neutrophilia or LN IFN- $\gamma$  production indicate that the absence of IL-4 from the transferred Th2 population does not instead result in Th1 priming, but rather a failure to generate a BSA-specific response.

## Discussion

To address whether Th2 priming to inhaled protein Ags is facilitated by ongoing Th2 inflammation and whether this priming could occur independently of the innate immune system, we used a murine model of ongoing pulmonary inflammation and evaluated the ability of naive T cells to become activated to a novel protein Ag. Following transfer of DO11.10-transgenic Th2 cells and inhaled Ag exposure (OVA), a nontransgenic, but OVA-specific, CD4<sup>+</sup> T cell population expands and is accompanied by the loss of the transferred Th2 cells (Fig. 2). This process was dependent on both the transfer of primed Th2 cells and the presence of endogenous naive CD4<sup>+</sup> T cells, as T cell-deficient mice were not capable of



**FIGURE 6.** Collateral Th2 sensitization to BSA is dependent on IL-4 production from transferred Th2 cells. A, BAL eosinophils, neutrophils, and lymphocytes from WT or IL-4 KO hosts receiving WT or IL-4 KO DO11.10 Th2 cells after primary challenge with OVA/BSA and secondary challenge with BSA alone. \*,  $p < 0.005$ ; \*\*,  $p < 0.02$ ,  $n = 6$ . B, IL-4, IL-5, and IL-13 (picograms per milliliter) production *ex vivo* by DLN cells from above groups. Cells were stimulated with APCs and 200  $\mu\text{g}/\text{ml}$  BSA. IFN- $\gamma$  was not detectable. One representative experiment of three is shown.

mounting a secondary response. Furthermore, if a second Ag (BSA) is introduced during initial activation of the transferred Th2 cells *in vivo*, a BSA-specific Th2 response is generated, as evidenced by pulmonary eosinophilic inflammation, BSA-specific Ab production, and lung DLN cell production of Th2 cytokines to BSA *ex vivo* (Figs. 3 and 4). Therefore, chronic Ag-specific Th2 inflammation can potentiate further naive T cell sensitization to distinct allergens in a process we have called collateral priming (Table I).

We have used the term collateral priming to describe the process of activation of naive T cells to their cognate Ag by adaptive immune signals to contrast it with the phenomena known as bystander activation (32–35). Although both processes involve activation of T cells by factors produced by a proximal but distinct T cell population, collateral priming is the activation of a naive T cell to its cognate Ag (as compared with an Ag-nonspecific response). Subsequently, this newly activated population can mediate Th2

Table I. Summary of conditions that result in collateral priming<sup>a</sup>

Host Immune Status	Protein Antigen Exposure	Host Response
Naive	Ag B	No Response
Immune to A	Ag B	No Response
Immune to A	Ag A	Immunity to A <sup>b</sup>
Immune to A	Ags A and B	Immunity to A and B <sup>b</sup>

<sup>b</sup> Independent of TLR4 and MyD88.

<sup>a</sup> Immunity to novel Ags, here Ag B (e.g., BSA or KLH), can develop during periods of inflammation elicited by other Ags, here Ag A (e.g., OVA), independent of TLR4 and MyD88.

inflammation in response to its Ag (e.g., BSA) independent of the T cells that originally activated them (e.g., the DO11.10 Th2 cells).

As previously reported, Th2 priming to an inhaled Ag in mice not undergoing an acute inflammatory reaction requires intact TLR4 signaling (7, 8) (Fig. 5A). In contrast, the process of collateral priming did not require TLR4 or MyD88, indicating that an adaptive immune response can bypass obligate innate immune signals in T cell priming (Fig. 5). Instead, IL-4 production by the initiating Th2 population plays a crucial role in the priming of a naive T cell population (Fig. 6). It is well established that IL-4 plays a central role in cytokine-directed Th2 differentiation and, using a similar system, it was recently shown that IL-4-producing Th2 cells can even overcome Th1-skewing adjuvants during Th2 differentiation to a new Ag (36). However, it has not been shown that IL-4 can influence naive T cell priming. In fact, IL-4 alone is insufficient to induce sensitization of Th2 cells; IL-2 is also required for initial clonal expansion, which is primarily induced after both APC signals have been delivered to the naive T cell (29, 37). This method of APC activation is dependent on the innate immune system recognition of non-self or other markers of inflammation, which has been clearly demonstrated to regulate the activation of naive T cells (6, 38). However, our studies clearly show that T cell priming can occur in the absence of innate immune system signals, as long as the adaptive arm of the immune system is activated. In the current model, we speculate that IL-4 from an OVA-specific Th2 cell may be playing a role in collateral priming by stimulating APCs to effectively present a simultaneously encountered Ag (BSA), as APC maturation is known to be an absolute requirement in T cell priming. Interestingly, allergic airway inflammation and IL-4 have both been shown to activate immature DCs in certain *in vivo* and *in vitro* models (39–41). Therefore, the identification of a Th2-priming pathway to inhaled protein Ags that involves adaptive rather than innate immune responses represents a novel mechanism by which helper T cells may become activated.

In the current studies, the two T cells involved have distinct Ag specificities; however, one could envision this process acting in an “autocrine” fashion to enhance the initial adaptive response to a single Ag. The outcome of these two effects would result in a more robust cellular immune response or “adaptive amplification.” Collateral priming may also represent a mechanism by which adaptive responses to one Ag are expanded to include distinct TCRs specific for other epitopes of the same Ag, thereby enhancing the magnitude and avidity of the T cell response (e.g., epitope spreading) (42). In addition, there are interesting correlations between collateral priming to inhaled protein Ags and a process termed heterologous immunity in which CD8<sup>+</sup> T cells specific for one virus respond to challenges with unrelated viruses and even facilitate clearance of the second virus (43).

The clinical implications of these findings relate to how asthmatics might acquire new sensitivities to environmental protein Ags during periods of chronic inflammatory processes. The natural course of allergic diseases such as asthma appears to involve the acquisition of new sensitivities, which is associated with an increase in overall IL-4 production from PBMC and IgE levels (44, 45). However, immunotherapy has been shown to block this acquisition of new allergies and can be accompanied by a reduction of PBMC proliferation and IL-4 production in response to specific allergens (11–13, 46–48). Therefore, the model presented here provides a possible mechanistic explanation of these clinical findings in that we have shown that IL-4 from an Ag-specific Th2 population facilitates further Th2 sensitization to new allergens. Furthermore, it suggests that immunotherapy or other treatments that can reduce IL-4 production and chronic Th2 inflammation in atopic patients could be effective in the long-term inhibition of

disease progression by interrupting the process of collateral priming (49, 50).

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