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Antigen-Nonspecific Recruitment of Th2 Cells to the Lung as a Mechanism for Viral Infection-Induced Allergic Asthma

Robin Stephens, David A. Randolph, Guangming Huang, Michael J. Holtzman, and David D. Chaplin

Respiratory viral infections have been shown to trigger exacerbations of asthma; however, the mechanism by which viral Th1-type inflammation exacerbates an allergic Th2-type disease remains unclear. We have previously shown that although adoptively transferred Th2 cells are inefficiently recruited to the lung in response to Ag, cotransfer of Th1 cells can increase accumulation of Th2 cells. In this study, we show that respiratory viral infection increases recruitment of resting Th2 cells specific for OVA even in the absence of OVA challenge. These findings suggest that the mechanism by which Th1-type inflammation enhances allergic asthma is via an effect on recruitment. To study the role of the antigenic specificity of Th1 cells in the enhancement of Th2 cell recruitment and to determine whether virus-induced recruitment of OVA-specific Th2 cells may involve Th1 cells specific to a different Ag, we tested whether hen egg lysozyme-specific Th1 cells could synergize with OVA-specific Th2 cells. Challenge of mice that had received adoptively transferred Th1 cells plus Th2 cells induced the expression of inflammatory chemokines in the lung and increased both recruitment and activation of Th2 cells, leading to eosinophil recruitment, even in the absence of challenge with the Th2 Ag. Interestingly, as IL-5 supports eosinophilia, culture of resting Th2 cells with fresh APC induced production of IL-5 in the absence of specific Ag. Thus, Ag-specific activation of Th1 cells enhances the recruitment potential of the lung leading to recruitment and activation of Th2 cells. This implies that circulating Th2 cells in allergic individuals could enter the lungs in response to infection or inflammation and become activated to trigger allergy.

For example, although IL-4 is a growth factor for Th2 cells, it can also inhibit the production of the Th1 cytokine IFN-γ (17). Although IFN-γ activates macrophages and directs B cells to class switch to the production of distinct Th1-specific Ab isotypes, it can also block switching to the Th2 isotypes, IgG1 and IgE, and can directly antagonize recruitment of eosinophils (18, 19). This cross-regulation, however, may not lead to exclusively polarized responses. Many responses are characterized by participation by both Th1 and Th2 cells and cytokines (e.g., the host response against malaria (20)), although one Th subset generally predominates in the response to a given Ag determining the nature of the inflammatory response and potentially the outcome of infection.

Our laboratory has previously observed that challenge of OVA-sensitized mice using an OVA aerosol leads to accumulation of Th1 cells in the lungs before accumulation of Th2 cells in the airways (7). Although the dominant immune effector functions associated with asthmatic inflammation are Th2 in character, we have observed that when Th2 cells are adaptively transferred into a naive mouse, airway challenge does not induce recruitment of the transferred Th2 cells into the airways. This suggests that Th2 cells are on their own poorly able to initiate a tissue inflammatory response when Ag is presented at low doses or for short time periods. This adoptive transfer model clearly showed that fully differentiated Th2 cells remain preferential producers of IL-4 or IFN-γ in vivo after transfer (Ref. 7, and see Fig. 6). Interestingly, when Th1 cells and Th2 cells are transferred together into a naive host, airway challenge induces the recruitment of both Th1 cells and Th2 cells to the airway, resulting in the accumulation of large numbers of eosinophils (21). Thus, the Th2 cells that on their own appear poorly competent to initiate a tissue inflammatory response are fully competent to enter the tissue and drive the local inflammatory response to one of Th2 character if the environment is prepared, in this case by Th1 cells. These findings substantiate the hypothesis that Th1 and Th2 responses are not exclusively counterregulatory.

Hospitalizations of patients with asthma exacerbations are often associated with viral or bacterial infections of the airways (22). In this context, the synergy that we have observed between the two Th subsets may be critical in the clinical setting. Asthma exacerbations are common in association with respiratory tract infections since the host immune response to viral infection is initiated and directed by Th1 cytokines such as IFN-γ. Similar results in mice show that infection with one of several viruses can exacerbate airway inflammation and hyperresponsiveness (23–25). These authors and others have proposed various mechanisms by which the tissue inflammation resulting from viral infections could lead to decreased lung function in asthmatics, but none of these accounts for the allergic nature of the asthma exacerbation (reviewed in Ref. 26). Our previous observation that Th1 cells can provide help for the recruitment of Th2 cells and consequently for the development of allergic inflammation led us to hypothesize that a major mechanism underlying the allergic phenotype of virally induced exacerbations of asthma could involve regulation of Th2 cell recruitment. This recruitment effect could depend on viral Ag-specific Th1 cells. Alternatively, it could be a manifestation of a generalized change in the airway environment that allows for nonspecific recruitment of immune cells.

The current experiments were designed to test whether respiratory virus infection could provide a stimulus for the recruitment of Th2 cells to the airways and to define the role of the T cell’s cognate Ag in this recruitment. Using OVA-specific transgenic Th2 cells, we show that respiratory infection with the mouse parainfluenza virus, commonly designated as Sendai virus, can stimulate recruitment of adoptively transferred Th2 cells to the lungs. Additional experiments using adoptive transfer of hen egg lysozyme (HEL)-specific Th1 cells along with OVA-specific Th2 cells demonstrate that recruitment of Th2 cells can be triggered by simultaneous Th1-type inflammation in the absence of the Th2 Ag. These experiments provide a mechanism by which the host response to respiratory virus infection may support the recruitment to the airways and activation of an unrelated population of Th2 cells. Furthermore, they demonstrate the Ag-nonspecific recruitment of Th2 cells to sites of tissue inflammation.

Materials and Methods

Recipient mice

Recipient mice were BALB/cAnHsd mice obtained from Harlan Breeders (Indianapolis, IN) were used between 6 and 8 wk of age. DO11.10 mice (27) that are transgenic for a Vα8.2 TCR that recognizes chicken OVA peptide 323–339 in the I-A d class II MHC protein were generously provided by K. Murphy (Washington University, St. Louis, MO). Thy1.1 DO11.10 mice were generated as previously described (21). For these experiments, the DO11.10 strain on the BALB/c background was crossed with B10.BR mice (The Jackson Laboratory, Bar Harbor, ME) to generate (DO11.10 × B10.BR)F1, mice. 3A9 mice (on the B10.BR background) that are transgenic for a Vβ8.2 TCR that recognizes HEL peptide 48–62 on I-A b (28) were a generous gift from E. Unanue (Washington University). For all of the experiments described here, these mice and B10.BR nontransgenic mice were crossed with BALB/cAnHsd (Harlan Breeders) to generate F1 mice. All mice were kept in microisolator cages in the specific pathogen-free facility of the Division of Comparative Medicine at the Washington University Medical Center. Experimental infections with Sendai virus were performed in a biohazard containment facility. Mice were supplied with sterile chow and water ad libitum. All animal procedures were approved by the Washington University Institutional Committee for the humane use of laboratory animals.

Culture and differentiation of Th cells

T cells were cultured in complete IMDM (T cell medium; cell components from Invitrogen, Carlsbad, CA) as previously described (27). Single-cell suspensions were prepared from the spleens of the DO11.10 and 3A9 mice using a disposable mesh (BD Falcon, San Jose, CA) and were cultured at 2.5–5 × 106 cells/ml with 0.3 μM OVA or 0.1 μM HEL peptide and the indicated cytokines. For production of Th2 cells, cultures contained 40 ng/ml recombinant murine IL-4 (R&D Systems, Minneapolis, MN) and a 1/8 dilution of anti-IFN-γ hybridoma supernatant (clone TOSH, provided by E. Unanue, Washington University). For preparation of Th1 cells, recombinant murine IL-12 10 ng/ml (BD PharMingen, San Diego, CA) and a 1/8 dilution of monoclonal anti-IL-4 supernatant (clone 11B11, provided by K. Murphy, Washington University) were added. Three days after initiating the cultures (day 3), the cells were split (1:4–1:6) and IL-2 (BD PharMingen) was added to 40 U/ml. On day 7, cells were split once again into fresh irradiated splenocytes with peptide. On day 10, the cells were split again and more IL-2 was added. Four days later (day 14), the cells were transferred to fresh medium and were “resting.” At this time they do not secrete cytokines without further stimulation with Ag or with PMA plus ionomycin (both from Sigma-Aldrich, St. Louis, MO), as assessed by intracellular staining and flow cytometry or by ELISA (R&D Systems) of culture supernatants. Resting but previously activated cells express intermediate levels of CD25 (29). Restimulation of the rested polarized cells with Ag and fresh APC led to expression of cytokines. Assessment of cytokine production by intracellular staining of monensin-, PMA-, and ionomycin-treated cells followed by flow cytometry showed that >30% of T cells in the Th1 cultures made IFN-γ, whereas <2% made IL-4 and 20–30% of cells in the Th2 cultures made IL-4, while >25% of Th2 cells in the Th1 experiments, cells were labeled with CFSE (Molecular Probes, Eugene, OR) by washing the cells in PBS without Ca2+ or Mg2+ (Invitrogen) and then incubating them at 1 × 106 cells/ml in 5 μM CFSE for 15 min. at 37°C in the dark. The labeling reaction was stopped by adding an equal volume of FCS and the cells were then washed thoroughly in PBS before adoptive transfer.

Adaptive transfer of differentiated cells and challenge with Ag

Differentiated, rested DO11.10 and 3A9 Th cells were transferred to naive mice i.v. via the retro-orbital plexus. The following day, 1% (w/v) Ag in PBS was administered via aerosol or intranasally. The aerosol was generated using a clinical nebulizer (UltraNeb 99; DeVilbiss Healthcare/Sunrise Medical, Sommerset, PA) which was calibrated to deliver droplets <4 μm in diameter and was administered to mice in a 15-inch square Plexiglas chamber as previously described (7). Aerosol treatment was for ~30 min and was repeated once 6–8 h later. For intranasal challenge, 40 μl of 1%
Ag was administered twice to mice anesthetized with methoxyflurane (Metofane; Schering-Plough Animal Health, Union, NJ). In experiments involving infection with Sendai virus, Th2 cells were administered on day 5 or day 8 following inoculation with virus. Aerosol or intranasal challenges were 1 day after T cell transfer and the mice were analyzed 3 days after Ag challenge. In all cases, aerosol and intranasal Ag challenges yielded results that were indistinguishable.

**Analysis of airway and lung leukocytes by flow cytometry**

Identification of adoptively transferred Th2 cells in isolated cell populations was performed by flow cytometry using CyChrome-conjugated clonotypic Ab for the DO11.10 transgene (KJ1-26-cy5; Caltag Laboratories, Burlingame, CA) (30) and the 3A9 transgene (1G12-biotin, a generous gift from E. Unanue and D. Peterson, Washington University). In selected experiments, the transgenic cells were identified by virtue of their common usage of a V/J82 TCR chain using anti-V/J82-Chyrome (Caltag Laboratories). Finally, where indicated, the transgenic Th2 cells were marked genetically with the allotypic marker Thy1.1 (and transferred into Thy1.2 BALB/c mice) or fluorescently with CFSE. Other fluorescent reagents included anti-CD25-PE, anti-CD4-CyChrome, anti-CD4-also-phycocyanin, and streptavidin-allophycocyanin (BD Pharmingen). Analysis was performed using a FACSCalibur with two lasers (red and 488 nm of argon) and CellQuest software (version 3.3; BD Immunocytochemistry Systems, Mountain View, CA). Intracellular staining for cytokines was achieved only after activating recovered cells with PMA (0.01 μg/ml) and ionomycin (1 μM) in the presence of 2 μM monensin (all from Sigma-Aldrich) for 4–6 h. The surface molecules were then stained as described and washed in 2% FCS. The cells were permeabilized by washing three times in PBS with 2% FCS containing 0.1 mg/ml saponin and stained for intracellular cytokines in this permeabilization buffer. Parallel cultures were stained with isotype control Abs (all from BD Pharmingen).

**Respiratory tract infection with Sendai virus**

Five hundred egg infectious units of Sendai virus were instilled into the nostrils of mice anesthetized with methoxyflurane. On the indicated day after intranasal infection, Thy 1.1– or CFSE-labeled DO11.10 Th2 cells were adoptively transferred i.v. Airway Ag challenge was 1 day after the adoptive transfer of Th2 cells by exposing mice to an aerosol of 1% OVA in PBS for 20 min or to 40 μl of 1% OVA in PBS administered intranasally. The mice were sacrificed and analyzed 3 days after Ag challenge.

**Analysis of chemokine RNA expression**

A total of 1.2 × 107 DO11.10 Th1 cells and/or Th2 cells was transferred i.v. into naive BALB/c recipients. One day later, these mice were challenged with a 30-min exposure to an aerosol of 1% OVA in PBS. Twelve hours later, their lungs were removed, frozen in liquid nitrogen, and total RNA was prepared using a Qiagen (Valencia, CA) miniprep kit according to the manufacturer’s instructions. Levels of chemokine RNA were determined using the Riboprint mammalian cDNA panel (BD Pharmingen) according to the manufacturer’s instructions.

**In vitro proliferation of transgenic T cells**

Splenocytes from DO11.10, 3A9, or nontransgenic mice (all on the BALB/c × B10.BR) background were cultured in triplicate at 2.5 × 106/ml in 96-well plates. OVA or HEL (10−5 or 10−6 M) was added, followed 5 days later by [3H]thymidine incorporation. Determined. Similar results were obtained on day 3, although the DO11.10 T cells showed maximal proliferation to the whole protein Ag later than did the 3A9 T cells. One micromolar OVA or 0.3 μM HEL peptide and medium alone provided positive and negative controls, respectively. In selected experiments, HEL (Sigma-Aldrich) that had been further purified by size exclusion chromatography to eliminate albumin (kindly provided by P. Allen, Washington University) was used for Ag challenge.

**Immunohistochemistry**

Paratracheal LN were frozen in OCT (Tissue-Tek, Torrance, CA) and 8-μm sections were fixed for 10 min in acetone and stained with KJ1-26-biotin followed by avidin multimer, Elite (ABC)-alkaline phosphatase and Vector Blue alkaline phosphatase substrate (Vector Laboratories) to observe the distribution of DO11.10 Th2 cells in the LN.

**Assessment of Ag-nonspecific IL-5 production**

Populations of resting DO11.10 or 3A9 Th1 or Th2 cells on a (B10.BR × BALB/c)F1 background were obtained 7 days after the last Ag challenge in vitro. CD4+ cells were purified from these cultures using CD4-specific Dynal magnetic beads (Dynal, Lake Success, NY) according to the manufacturer’s instructions. At this point the purified Th1 and Th2 cells were negative by intracellular FACS staining or 72-h ELISA for IFN-γ or IL-4 unless they were stimulated again with PMA and ionomycin. A total of 2.5 × 104 purified (B10.BR × BALB/c)F1 TCR transgenic Th1 or Th2 cells was cultured together with 5 × 105 irradiated splenocytes from (B10.BR × BALB/c)F1 mice in 1 ml of T cell medium. Supernatants were recovered after culture for 72 h under 8% CO2 and analyzed by ELISA (R&D Systems) for the presence of IL-5 in the supernatant.

**Results**

**Infection with Sendai virus increases recruitment of Th2 cells to the airways**

To investigate the relationship between respiratory virus infection and recruitment of Th2 cells to the airway after Ag challenge, we analyzed the effect of acute parainfluenza virus infection on the recruitment of adoptively transferred Th2 cells in otherwise naive mice. Animals infected with 500 egg infectious doses of Sendai virus (~1/10 the LD50) did not become fatally ill. They did, however, show evidence of significant inflammation in the airways. There was a large influx of monocytes, macrophages, and neutrophils observed by Wright’s staining of the bronchoalveolar lavage (BAL) cells. Analysis by flow cytometry and intracellular cytokine staining also showed that 50% of the infiltrating endogenous CD4+ T cells were capable of producing IFN-γ. These data indicated that there was a significant Th1-type inflammatory response to Sendai virus infection. Infected mice (BALB/c, Thy1.2) were given 107 OVA-specific DO11.10 Thy1.1 Th2 cells at the peak of virus infection (days 5–8). One day later, the infected mice were exposed to an aerosol of 1% OVA. Three days after this challenge, the airways were sampled by BAL. The transferred Th2 cells were identified by surface staining of the BAL cells for the allotypic marker Thy1.1 and phenotyped by intracellular cytokine staining and flow cytometry as Th2 on the basis of their production of IL-4 but not IFN-γ. Mice infected with Sendai virus showed recruitment of a larger number of Thy1.1+, IL-4+, IFN-γ−cells compared with uninfected mice (Fig. 1). Thus, infection with a sublethal dose of Sendai virus enhanced the recruitment of the OVA-specific Th2 cells. Interestingly, a 4-fold increase in the numbers of recruited eosinophils was also observed in the infected mice challenged with OVA compared with mice that were challenged with OVA alone (Fig. 1A). Together, these data suggested that the antiviral (Th1-type) response could occur simultaneously with the allergic inflammatory (Th2-type) response. In fact, the antiviral response appeared to enhance Th2 cell recruitment and not to preclude Th2 effector functions.

To test whether the recruitment of the Th2 cells that was induced by virus infection was dependent on challenge with the cognate Th2 cell Ag, we analyzed the number of OVA-specific Th2 cells in the BAL after virus infection with or without aerosol OVA challenge (Fig. 1B). Interestingly, virus infection resulted in a dramatic increase in the number of OVA-specific Th2 cells that could be recovered by BAL even without OVA challenge. Infection without OVA challenge induced airway inflammation equivalent to that seen in mice infected with virus and then subjected to airway Ag challenge. The numbers of Th2 cells recruited to the airways in the presence of an antiviral response approach the numbers obtained when Th1 and Th2 cells were transferred together before challenge with OVA. This observation indicated that these Th2 cells could be recruited to the airways in an Ag-independent fashion.

The Ag-independent recruitment of Th2 cells to the lungs seen in the context of respiratory viral infection did not represent a global, systemic mobilization of Th2 cells. Although transgenic...
cytes gated on CD4 were analyzed by flow cytometry as CD4+Thy1.1+ DO11.10 Th2 cells were transferred into naive or infected BALB/c mice (Thy1.2). The following day, mice were challenged twice for 30 min with an aerosol of 1% OVA in PBS. On the third day after challenge, airway cells were collected by BAL. BAL cells from five replicate mice were pooled and analyzed for the presence of transferred Th2 cells (identified by flow cytometry as CD4+Thy1.1+IL-4+IFN-γ+). Eosinophils were counted in a cytoospin preparation of the BAL cells using Wright’s stain. Flow cytometry as CD4+Thy1.1+DO11.10 cells were stained for the DO11.10 clonotype, KJ1-26.

To test the hypothesis that a local Th1 response of any antigenic specificity might trigger recruitment of circulating allergic Th2 cells, we substituted transgenic HEL-specific Th1 cells for the virally induced inflammatory response. In this double transgenic adoptive Th cell transfer system, the Ag specificity of the Th1 cells was different from that of the Th2 cells, allowing the two Th subsets to be activated independently. This also allowed for investigation of the role of antigenic specificity in recruitment of T cells. Although it is widely accepted that innate cells are recruited in an Ag-nonspecific fashion, this has not been previously established for the Ag-specific cells of the immune system. DO11.10 Th2 cells recognizing OVA were adoptively transferred into naive mice along with 3A9 Th1 cells recognizing HEL. Since both 3A9 and DO11.10 T cells express Ag receptors using the Vβ8.2 chain, the transferred Th2 cells that localized to the lung could be identified by flow cytometry as surface Vβ8.2+ and intracellular IL-4+IFN-γ+. The Vβ8.2 gene is expressed by ~5–10% of all circulating T cells (depending on the strain), introducing some background into the determination of OVA- and HEL-specific cells; however, use of this marker along with anti-CD4, anti-IL-4, and anti-IFN-γ Abs permitted us to measure intracellular cytokines. The cytokine staining allowed us to identify clearly that the transferred cells were previously activated. In other experiments, we have shown that adoptively transferred Th1 cells do not switch phenotype under these conditions in vivo (7, 21). Since genetic background can affect studies of Th1 and Th2 cells in some systems (31) and since the DO11.10 and 3A9 TCR transgenes required that the experiment be performed using the H-2d×H-2k ((BALB/c×B10.BR)F1) background, we first tested whether this mixed genetic system showed cooperation for recruitment between Th1 and Th2 cells as we had seen in our earlier studies (7, 21). Using either DO11.10 or 3A9 Th2 cells, we found that few Th2 cells were recruited into the lungs of BALB/c×B10.BR recipients unless Ag-stimulated Th1 cells were also recruited to the tissue (Fig. 2, A and B). Thus, the (BALB/c×B10.BR)F1 system yielded similar results compared with the BALB/c system. Interestingly, although we confirmed that Th1 cells are recruited to the airway in response to aerosolized Ag when adoptively transferred without Th2 cells, we found that the numbers of Th1 cells that were recruited was increased when Th2 cells were also present. This suggests that perhaps in reciprocal fashion Th2 cells can provide signals supporting enhanced Th1 cell recruitment.

Before testing whether it was necessary for both the Th1 cells and the Th2 cells to be stimulated by Ag to show cooperation for Th2 cell recruitment, we excluded the possibility that these cells might show cross-responsiveness to each Ag preparation. Analysis

FIGURE 1. Respiratory virus infection drives recruitment of Th2 cells to the airway. BALB/c mice were infected intranasally with 500 egg infectious units of Sendai virus. A. Eight days after infection, 10^7 Thy1.1+ DO11.10 Th2 cells were transferred into naive or infected BALB/c mice (Thy1.2). The following day, mice were challenged twice for 30 min with an aerosol of 1% OVA in PBS. On the third day after challenge, airway cells were collected by BAL. BAL cells from five replicate mice were pooled and analyzed for the presence of transferred Th2 cells (identified by flow cytometry as CD4+Thy1.1+IL-4+IFN-γ+). Eosinophils were counted in a cytoospin preparation of the BAL cells using Wright’s stain. B. Five days after infection, 10^7 CFSE-labeled DO11.10 Th2 cells were transferred into naive or infected BALB/c mice. The following day, mice were challenged with 1% OVA as above. On the third day after challenge, BAL cells from five replicate mice were pooled and analyzed for the presence of transferred Th2 cells (identified by flow cytometry as CD4+KJ1-26+CFSE+). The numbers of recruited Th2 cells were calculated as the product of the percent Th2 cells in a pooled BAL sample for each group and the total number of BAL cells in each sample. C. Airway Ag leads to proliferation of Th2 cells in the local LN. Recruitment and proliferation of adoptively transferred Th2 cells were assessed in the draining (paratracheal) and nondraining (inguinal) LN. Using mice from the experiment shown in B, the LN were dissociated to single-cell suspensions and analyzed by flow cytometry. The dot plots each represent ~10,000 lymphocytes gated on CD4+ cells and stained for the DO11.10 clonotype, KJ1-26. The percentages of KJ1+CFSEhigh and CFSElow cells relative to total CD4+ cells are shown. This experiment was repeated three times (n = 5).

OVA-specific Th2 cells were found in large numbers in BAL recovered from mice infected with Sendai virus but not challenged with OVA, paratracheal LN of mice infected with Sendai virus but not challenged with OVA showed a percentage of DO11.10 Th2 cells similar to that of nondraining inguinal LN (Fig. 1C). To determine whether the increase in cell number seen in the lungs was due to increased recruitment to the airways or to Ag-independent proliferation of the Th2 cell population, we tested the adoptively transferred cells for evidence of cell division. To permit analysis of cell division, the Th2 cells were labeled with CFSE before adoptive transfer. Proliferation of the adoptively transferred Th2 cells was monitored using flow cytometry by observing the decrease in intensity of CFSE fluorescence that occurs when labeled cells proliferate. Evidence of Th2 cell proliferation was detected in cells that were localized in the paratracheal LN 3 days after OVA challenge of Sendai virus-infected mice. Only those mice that received an OVA aerosol challenge showed an increased percentage of Th2 cells in the paratracheal LN. Transgenic cells recruited to this tissue showed diminished CFSE fluorescence indicating that they had proliferated. Although the percentage of nonproliferating KJ1-26+ cells in the LN did not vary greatly between experimental groups, the percentage of proliferating cells did increase in the presence of Ag (Fig. 1C). Thus, proliferation of Th2 cells in the regional node was Ag dependent. Importantly, however, even when proliferation did not occur in the local LN, Th2 cells could still localize efficiently to the airways in response to respiratory virus infection.

Matching Ag specificity is not required for the increase in Th2 recruitment elicited by cooperating Th1 cells

To test the hypothesis that a local Th1 response of any antigenic specificity might trigger recruitment of circulating allergic Th2 cells, we substituted transgenic HEL-specific Th1 cells for the virally induced inflammatory response. In this double transgenic adoptive Th cell transfer system, the Ag specificity of the Th1 cells was different from that of the Th2 cells, allowing the two Th subsets to be activated independently. This also allowed for investigation of the role of antigenic specificity in recruitment of T cells. Although it is widely accepted that innate cells are recruited in an Ag-nonspecific fashion, this has not been previously established for the Ag-specific cells of the immune system. DO11.10 Th2 cells recognizing OVA were adoptively transferred into naive mice along with 3A9 Th1 cells recognizing HEL. Since both 3A9 and DO11.10 T cells express Ag receptors using the Vβ8.2 chain, the transferred Th2 cells that localized to the lung could be identified by flow cytometry as surface Vβ8.2+ and intracellular IL-4+IFN-γ+. The Vβ8.2 gene is expressed by ~5–10% of all circulating T cells (depending on the strain), introducing some background into the determination of OVA- and HEL-specific cells; however, use of this marker along with anti-CD4, anti-IL-4, and anti-IFN-γ Abs permitted us to measure intracellular cytokines. The cytokine staining allowed us to identify clearly that the transferred cells were previously activated. In other experiments, we have shown that adoptively transferred Th1 cells do not switch phenotype under these conditions in vivo (7, 21). Since genetic background can affect studies of Th1 and Th2 cells in some systems (31) and since the DO11.10 and 3A9 TCR transgenes required that the experiment be performed using the H-2d×H-2k ((BALB/c×B10.BR)F1) background, we first tested whether this mixed genetic system showed cooperation for recruitment between Th1 and Th2 cells as we had seen in our earlier studies (7, 21). Using either DO11.10 or 3A9 Th2 cells, we found that few Th2 cells were recruited into the lungs of BALB/c×B10.BR recipients unless Ag-stimulated Th1 cells were also recruited to the tissue (Fig. 2, A and B). Thus, the (BALB/c×B10.BR)F1 system yielded similar results compared with the BALB/c system. Interestingly, although we confirmed that Th1 cells are recruited to the airway in response to aerosolized Ag when adoptively transferred without Th2 cells, we found that the numbers of Th1 cells that were recruited was increased when Th2 cells were also present. This suggests that perhaps in reciprocal fashion Th2 cells can provide signals supporting enhanced Th1 cell recruitment.
were adoptively transferred independently or together into naive (BALB/c/H11003 Th2 cells. The 3A9 and DO11.10 transgenes were each bred onto a (BALB/c/H9252 cells were distinguished by their staining for surface Vβ8.2 (the β-chain of both the DO11.10 and the 3A9 TCR) and intracellular IFN-γ (for Th1 cells) and intracellular IL-4 (for Th2 cells). Th1 cells presented in A and B were identified as CD4+ Vβ8.2+ IL-4+ IFN-γ−, whereas Th2 cells are CD4+ Vβ8.2+ IL-4+ IFN-γ+. Similar data were obtained using the clonotypic Ab KJ1-26 that recognizes the transgenic DO11.10 TCR (as seen in Fig. 3, left panel). Airway eosinophils were detected by Wright’s stain of cytospin preparations. This experiment was repeated four times with similar results. C. Lack of cross-reactivity between 3A9 and DO11.10 T cells. Freshly isolated 3A9 (■), DO11.10 (□), or nontransgenic (□) (B10.BR × BALB/c)F1 splenocytes were plated in triplicate at 2.5 × 10^6 cells/ml in T cell medium with either 10^−6 M or 10^−5 M HEL or OVA protein as indicated. On day 4 of culture, [3H]thymidine (1 μCi) was added and 24 h later cells were harvested and [3H]thymidine incorporation was determined. Data shown are the mean ± SD of triplicate wells. Similar results (although less absolute proliferation) were obtained if the cultures were harvested 3 days after stimulation. In addition, similar data were obtained if stimulation was with synthetic HEL (48–62) and OVA (323–339) peptides or using repurified OVA and HEL as described in Materials and Methods. This experiment was repeated twice.

of the proliferative response of splenocytes from 3A9 mice and DO11.10 mice to two doses of either OVA or HEL showed that proliferation was limited to the appropriate cognate Ag (Fig. 2C). Having demonstrated that both DO11.10 and 3A9 Th1 and Th2 cells could cooperate in the (BALB/c × B10.BR)F1 genetic background, we then tested whether Th1 and Th2 cells of different antigenic specificities could affect each other’s recruitment in the same way that virus infection or OVA-specific Th1 cells influenced OVA-specific Th2 cell accumulation (Fig. 3). Th1 and Th2 cells derived from transgenic mice encoding two different antigenic specificities were transferred into unprimed syngeneic mice and various combinations of the Th1 and the Th2 Ags were administered via aerosol. In this experiment, the OVA-specific DO11.10 Th cells were identified in the BAL using anti-CD4 plus the clonotypic Ab KJ1-26 and the HEL-specific 3A9 Th cells were identified using anti-CD4 plus the 1G12 mAb. Although this allowed us to identify each population of transgenic cells specifically, it did not permit measurement of intracellular cytokines using a four-color FACS machine. In previous experiments, however, we showed that Th1 and Th2 cells retain their starting phenotype over the course of experiments such as these. When HEL and OVA were administered together to recipients of a mixture of DO11.10 Th2 cells and 3A9 Th1 cells, substantial numbers of Th2 cells were recruited (Fig. 3). Thus, the ability of Th1 cells to provide stimuli for the recruitment of Th2 cells is not dependent on both cell types recognizing the same Ag.

To test whether there was any absolute requirement for the Th2 Ag to elicit the recruitment of Th2 cells to the airways, mice that had received adoptively transferred Th1 cells and Th2 cells of different antigenic specificity were treated with an aerosol of only the Ag specific for the Th1 cells. Similar to the results with respiratory viral infection, treatment with the aerosolized Th1 Ag was sufficient to drive the recruitment to the airways of both Th1 cells (specific for the eliciting Ag) and Th2 cells (specific for an Ag unrelated to the eliciting Ag). This was true whether the Th1 Ag was HEL or OVA (Fig. 3, left panel and right panel, respectively). Although in the experiment shown here the number of eosinophils recruited to the BAL was higher when recruited by 3A9 Th2 cells, this was an inconstant finding that was not reproduced in replicate experiments. Recruitment of Th2 cells to the airways in response to an Ag that was distinct from their own cognate Ag suggested that the challenge Ag or the Th1 response it elicited enhanced, in a nonspecific fashion, the recruitment functions within the tissue for lymphocytes in general. To investigate this further, we tested the effects of Ag challenge and Th1 cell recruitment on expression of chemokine genes in the lung.

**Recruitment of Th1 cells to the lungs is associated with up-regulation of chemokine expression**

Our observation that Th2 cell recruitment to the lungs and airways can occur without the Th2 Ag is consistent with a model in which the primary stimulus for Th2 cell recruitment is the triggering by locally produced inflammatory mediators of adhesive and chemoattractant signals that render the tissue receptive for recruitment of leukocytes circulating in the bloodstream. Because local recruitment of Th1 cells can potentiate Th2 cell recruitment, we next investigated the nature of the signals that aerosolized Ag and recruited Th1 cells elicited in the tissue that might contribute to

**FIGURE 2.** Th1 and Th2 cells can cooperate for Th2 cell recruitment to the airways. A. Recruitment of DO11.10 Th1 cells provides help for the recruitment of DO11.10 Th2 cells on the (BALB/c × B10.BR)F1 background. B. Recruitment of 3A9 Th1 cells provides help for the recruitment of 3A9 Th2 cells. The 3A9 and DO11.10 transgenes were each bred onto a (BALB/c × B10.BR)F1 background. Th1 (10^7) and/or Th2 (10^7) cells prepared from the HEL-specific 3A9 TCR transgenic mice (3A9) and the OVA-specific DO11.10 TCR transgenic mice (DO) on the (BALB/c × B10.BR)F1 background were adoptively transferred independently or together into naive (BALB/c/H11003 Th2 cells. The 3A9 and DO11.10 transgenes were each bred onto a (BALB/c × B10.BR)F1 background. Th1 (10^7) and/or Th2 (10^7) cells prepared from the HEL-specific 3A9 TCR transgenic mice (3A9) and the OVA-specific DO11.10 TCR transgenic mice (DO) on the (BALB/c × B10.BR)F1 recipient animals. One day after adoptive transfer, aerosol Ag challenge using a mixture of 1% HEL plus 1% OVA in PBS for all groups was performed twice, 6 h apart, and 3 days later cells recruited to the airway were collected by BAL. BAL cells from two to four mice per group were pooled, stained, and analyzed by flow cytometry. The transgenic Th1 and Th2 cells were distinguished by their staining for surface Vβ8.2 (the β-chain of both the DO11.10 and the 3A9 TCR) and intracellular IFN-γ (for Th1 cells) and intracellular IL-4 (for Th2 cells). Th1 cells presented in A and B were identified as CD4+ Vβ8.2+ IL-4+ IFN-γ−, whereas Th2 cells are CD4+ Vβ8.2+ IL-4+ IFN-γ+. Similar data were obtained using the clonotypic Ab KJ1-26 that recognizes the transgenic DO11.10 TCR (as seen in Fig. 3, left panel). Airway eosinophils were detected by Wright’s stain of cytospin preparations. This experiment was repeated four times with similar results. C. Lack of cross-reactivity between 3A9 and DO11.10 T cells. Freshly isolated 3A9 (■), DO11.10 (□), or nontransgenic (□) (B10.BR × BALB/c)F1 splenocytes were plated in triplicate at 2.5 × 10^6 cells/ml in T cell medium with either 10^−6 M or 10^−5 M HEL or OVA protein as indicated. On day 4 of culture, [3H]thymidine (1 μCi) was added and 24 h later cells were harvested and [3H]thymidine incorporation was determined. Data shown are the mean ± SD of triplicate wells. Similar results (although less absolute proliferation) were obtained if the cultures were harvested 3 days after stimulation. In addition, similar data were obtained if stimulation was with synthetic HEL (48–62) and OVA (323–339) peptides or using repurified OVA and HEL as described in Materials and Methods. This experiment was repeated twice.
Th1 and Th2 cells were prepared from 3A9 and DO11.10 (DO) transgenic mice on the (BALB/c × B10. BR/F1) background and, as indicated, 10^7 of each were adoptively transferred into naive (BALB/c × B10. BR/F1) recipients. The mice were challenged 1 day later with aerosolized HEL and/or OVA (1% in PBS) as indicated. Three days later, the numbers of recruited transgenic Th1 and Th2 cells were determined by flow cytometry of BAL cells using anti-CD4 and anti-TCR clonotypic Abs (clone 1G12 for 3A9 and KJ1-26 for DO). Eosinophils were detected by Wright’s stain of cytopsin preparations. The right and left panels represent reciprocal Th1 and Th2 cell specificities. Pooling BAL cells from replicate mice (n = 2–3) in each group was necessary to provide sufficient numbers of cells for staining and FACS. Similar results were obtained in four different experiments.

subsequent homing of Th2 cells to the airway, DO11.10 Th1 and/or Th2 cells were transferred into naive BALB/c mice and the mice were subsequently challenged with aerosol OVA. Twenty-four hours (Fig. 4) or 36 h (data not shown) after challenge, the lungs were collected and total lung RNA was analyzed for the presence of chemokine transcripts using an RNase protection assay. Twenty-four hours after OVA challenge of naive mice that received no adoptively transferred cells, there was only low, baseline expression of RANTES and very low expression of eotaxin-1, macrophage-inflammatory protein (MIP) 1β, MIP-1α, and MIP-2. These levels of expression were indistinguishable from those of mice receiving no Ag challenge (data not shown). Mice that had received only OVA-specific Th2 cells showed only small increases in Ag-stimulated chemokine RNA levels. In contrast, mice that received OVA-specific Th1 cells or Th1 cells plus Th2 cells showed a dramatic increase in expression of the tested chemokines. Several of the chemokines up-regulated in association with Th1 cell recruitment, including eotaxin (32), RANTES, and MIP-1α (33), have been shown to support the development of eosinophilic airway inflammation in a coordinated sequential fashion. In the course of investigating the expression of other molecules that participate in leukocyte recruitment, we have previously reported that VCAM-1 is up regulated in association with Th1 cell recruitment and that administration of blocking Abs to VCAM-1 limits the ability of Th1 cells to cooperate in the recruitment of Th2 cells and eosinophils (21). In this study, we report that when Th1 cells are recruited to the airways, there is increased expression of several chemokines that may contribute to establishing a milieu that is permissive for subsequent Ag-independent recruitment of Th2 cells to the lungs.

Local Ag challenge is required for accumulation of Th2 cells in the regional LN

To begin to test whether trafficking of Ag specific Th2 cells through the LN is needed for these cells to acquire the potential to migrate to peripheral tissues, we investigated whether recruitment of Th2 cells to the lungs and airways was associated with localization to the paratracheal LN. The paratracheal LN from mice that had received DO11.10 Th2 cells and/or 3A9 Th1 cells before aerosol Ag challenge with combinations of HEL and/or OVA were analyzed immunohistochemically for the presence of DO11.10 Th2 cells using the KJ1-26 Ab (Fig. 5). We observed first that there were many Ag-specific Th2 cells in these draining LN after Ag challenge, whether or not this challenge was sufficient to drive Th2 cell recruitment to the lungs. For example, when DO11.10 Th2 cells were transferred along with 3A9 Th1 cells and challenge was with OVA alone, there were many Th2 cells in the local LN (Fig. 5C) even though under these conditions there was little recruitment of either Th1 or Th2 cells to the lungs (Fig. 3). The pattern of Th2 cell accumulation in the regional LN was similar in mice that were challenged with both OVA and HEL (Fig. 5A), conditions under which there was robust recruitment of Th2 cells to the airways (Fig. 3). As in mice infected with Sendai virus but not receiving aerosol Ag challenge (Fig. 1C), recruitment of Th2 cells to the airways did not appear to be dependent on accumulation of Th2 cells in the draining LN. This recruitment of Th2 cells to the airway without accumulation in the draining LN is consistent with the recent data of Gajewski et al. (34) suggesting that Th2 predominant experimental allergic airway inflammation can develop in mice with congenital absence of peripheral LN.

Th2 cells can acquire a partially activated phenotype even in the absence of their cognate Ag

Ag-nonspecific recruitment of Th2 cells to the lung may have very little impact on the tissue if the recruited cells are not activated. In

![FIGURE 3.](image1.png)  

![FIGURE 4.](image2.png)
contrast, Ag-nonspecific Th2 cell recruitment may elicit the full gamut of Th2-driven pathologic changes if the recruited Th2 cells manifest an activated phenotype. In this regard, it is of interest that in mice that received HEL-specific Th1 cells and OVA-specific Th2 cells followed by challenge with the Th1 Ag alone there was recruitment of not only the OVA-specific Th2 cells, but also substantial numbers of eosinophils (Fig. 4). This was despite the fact that the adoptively transferred Th cells had reverted to a resting phenotype before their infusion in vivo. Given that the Th2 cytokine IL-5 seems to be required for mobilization of eosinophils in murine eosinophilic airway inflammation (35, 36), our findings suggest that recruitment of Th2 cells to the lung, even in the absence of the Th2 cell Ag, may result in activation of the cells to make IL-5 that may then support the mobilization and survival of eosinophils. These mice also showed increased mucus production as detected by periodic acid-Schiff staining (data not shown), representing an additional Th2 effector function (8, 37, 38).

The level of activation of cells that had been recruited to the airways was examined further by flow cytometry of the BAL lymphocytes for the level of expression of CD25. In vitro expression of CD25 is elevated on primary Th cells from day 2 or 3 after challenge with Ag and remains high until day 5 or 6 when it is reduced to an intermediate level of surface expression. Ag-experienced cells remain CD25^int for weeks but are induced again for several days following restimulation, up to two orders of magnitude in relative fluorescence units, to the high levels seen following the initial activation (39, 40). Surprisingly, in the BAL from Ag-challenged mice, similar numbers of Th2 cells expressed high levels of CD25 whether the airways were challenged with the cognate Th2 Ag or with a nonspecific Ag (Fig. 6). Similar CD25 expression was observed on Th2 cells recruited in mice that had received DO11.10 Th2 cells (Fig. 6) or 3A9 transgenic cells (data not shown). Because resting, CD25^int Th2 cells do not enter the BAL in this system in the absence of Th1 cells and since in the presence of Th1 cells they acquire an activated phenotype, it is not possible to recover CD25^int, previously activated but not presently activated, Th2 cells in the BAL as a control; however, the levels of CD25 on Th2 cells recovered from BAL of mice that had been treated with either a combination of Th1 and Th2 Ags or with the Th1 Ag alone are substantially higher than those seen on resting cells in vitro before adoptive transfer. Taken together, the findings that Th2 recruitment was accompanied by recruitment of eosinophils and also by increased expression of CD25 on Th2 cells recovered from the BAL indicate that recruitment of Th2 cells mediated by Th1 cells and Th1 Ag was accompanied by at least partial activation of the recruited Th2 cells.
**FIGURE 6.** Th2 cells recruited to the lungs manifest an activated surface phenotype even in the absence of the Th2 cell Ag. HEL-specific 3A9 Th1 cells and OVA-specific DO11.10 Th2 cells (10^5 each) were adoptively transferred into naive recipients. One day later, aerosol challenge was performed twice 6 h apart with 1% HEL plus 1% OVA (left panel) or 1% HEL alone (right panel). Three days after challenge, cells harvested by BAL from duplicate mice were pooled and analyzed by flow cytometry, gating on CD4^+ cells and analyzing staining with the DO11.10 clonotypic Ab KJ1-26 and with the activation marker IL-2R (CD25). Data plotted represent CD4^+ cells from a total of 10,000 events collected in the lymphocyte gate. Similar results were obtained in one additional experiment.

**Discussion**

An association between respiratory viral infections and exacerbations of asthmatic inflammation has been appreciated for several decades. Although virus infection of airway epithelial cells can lead to desquamation exposing nerve endings and to other changes predisposing to bronchial hyperreactivity, this explains only a portion of the pathologic response. The full cascade of events leading from virus infection to exacerbation of allergic inflammation in human asthmatics remains poorly understood. In the experiments presented here, we have shown that respiratory virus infection can increase recruitment of Th2 cells to the airway, providing a potential mechanism for promotion of Th2-type inflammation in response to virus in individuals with an underlying Th2 diathesis.

In general, the immune response to virus is Th1-like in its effector mechanisms. These mechanisms include, in addition to the antiviral Th1 cells of the adaptive response, Ag-nonspecific elements recruited from the innate immune response. There is a large body of data showing cross-interference of Th1-type and Th2-type responses. This can be observed both at the level of phenotype differentiation and at the level of expression of effector functions. Many studies have suggested that soluble mediators produced in the course of Th1-type and CD8-type responses could inhibit effector functions of coexisting Th2 cells. Although cross-interference of Th1-type and Th2-type responses is broadly accepted, there are also examples, particularly in models of experimental infection, of both types of responses being launched in a single host against a single pathogen, resulting in a composite response of mixed phenotype.

In this study, we have extended our previous findings (7, 21) that Ag-specific Th1 cells can potentiate Th2 cell recruitment and the resulting Th2-like tissue inflammation. We have used respiratory tract infection with Sendai virus as an experimental model in mice for the respiratory tract infections that are associated with exacerbations of asthma in human patients. In this model, the antigenic specificity of the antiviral response is unlikely to be related to the antigenic specificity of the pathologic Th2 cells that ultimately lead to the allergic inflammation. In a parallel system, we

**Table 1. Ag-nonspecific production of IL-5 by transgenic Th2 cells**

<table>
<thead>
<tr>
<th>Transgenic T Cells</th>
<th>DO Th1, 3A9 Th2</th>
<th>3A9 Th1, DO Th2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/ml IL-5 (±SD)</td>
<td>7.0 (±11.00)</td>
<td>7.0 (±6.7)</td>
</tr>
<tr>
<td>Th2 plus APC/no Ag</td>
<td>42.9 (±13.00)</td>
<td>151.0 (±48)</td>
</tr>
<tr>
<td>Th2 plus APC/Age</td>
<td>1015.9 (±59.00)</td>
<td>942.2 (±56.3)</td>
</tr>
</tbody>
</table>

*To determine whether stimulation of Th2 cells by their cognate Ag is required for their production of IL-5, we cocultured CD4-purified, washed BALB/c × B10.BR 3A9 Th2 cells ± DO11.10 Th1 cells (left column) or DO11.10 Th2 cells ± 3A9 Th1 cells (right column) along with irradiated syngeneic splenocytes with or without the cognate Th2 cell peptide Ag. After 72 h, the culture supernatant was tested for IL-5 by ELISA. Data represent the means ± SD of triplicate determinations. Similar data were obtained with supernatants harvested after 36 h. This experiment was repeated twice with multiple time points.*

*Peptide added to resting T cells in the absence of added APC.*

*Th1 cells included.*
have used HEL-specific transgenic Th1 cells as another way of providing a Th1-like inflammatory signal in the context of an unrelated Th2 response (here directed against the model Ag, OVA). In both systems, we have shown that Th1-like responses, irrespective of the Ag that elicited them, can synergize with Th2 cells to promote eosinophilic airway inflammation. This synergy is expressed largely at the level of facilitating recruitment of the Th2 cells to the tissue.

Because recruitment of adoptively transferred Th1 cells leads to an increase in both airway inflammation and in recruitment of Th2 cells into the lung, we considered that Th1-specific cytokines might be the primary signals supporting the recruitment of Th2 cells; however, efforts to block signals delivered uniquely by Th1 cells by administration of a neutralizing anti-IFN-γ Ab, a neutralizing lymphotixin β receptor-Ig fusion protein, or a neutralizing anti-IL-2Rα Ab did not inhibit recruitment of Th2 cells in mice that had received cotransfer of Th1 and Th2 cells (Ref. 21 and our unpublished data). Thus, not one of these Th1 cell products was dominantly responsible for the cooperation observed between Th1 and Th2 cells (Ref. 21 and our unpublished data). This leads to up-regulation of IL-5 expression as measured by ELISA of culture supernatants (Table I). Future studies will investigate the nature of the activating signal that is provided by the APC. Several previous studies have reported Ag-independent activation of human CD4+ T cells in vitro. Treatment with a combination of the cytokines IL-2, IL-6, and TNF has been shown to stimulate human T cells to proliferate and secrete IL-2 (42). Furthermore, culture of differentiated Th2 cells with IL-10 has been shown to increase transcription of IL-4, IL-9, and IL-13 (43), and culture of Th1 cells with a mixture of IL-12 and IL-18 has been shown to induce production of IFN-γ without stimulation of the TCR (44). Additional studies will be required to determine whether any of these signals contributes to the Ag-independent activation of Th2 cells that we have observed here. Regardless of the mechanism by which coculture with APC induces IL-5 expression, we suggest that perhaps similar signals can be delivered in vivo as the recruited Th2 cells encounter tissue APC or cells with similar activating potential. The Ag-independent activation that occurs in vivo may be limited to cytokine secretion and may represent partial activation as originally described by Evavold et al. (44). Alternatively, it may include activation of all effector responses. In experiments using infection with Sendai virus to mobilize Ag-independent Th2 cell recruitment, we observed a moderate level of tissue eosinophilia but with no evidence of proliferation of Th2 cells, suggesting that in this case the Ag-independent Th2 cell activation was indeed incomplete.

The type of Ag-nonselective local activation of T cells that we have observed here would have grave implications for patients with large numbers of circulating allergen-specific Th2 cells. Such nonselective activation would place an atopic individual at risk for a flare of allergic inflammation whenever a local tissue injury occurred. We suspect that this underlies the reported coincidence of respiratory tract infections and clinically significant asthma exacerbations.

Our data demonstrate that the T cell Ag does not play a defining role in T cell recruitment to the airways; however, T cell Ag does lead to proliferation in the LN. We suspect that this action of Ag is a major regulatory one in the majority of T cell-dependent tissue responses in vivo. Under normal conditions, the numbers of Ag-specific T cells are carefully regulated so that large numbers of Ag-specific T cells are present only when a brisk local or systemic response is required. Thus, in the normal setting when the numbers of Ag-experienced cells are low, nonspecific recruitment of T cells to a site of tissue inflammation might result in only limited inflammatory potential. Ag, driving the recruitment of specifically reactive T cells to the regional nodes to permit clonal expansion under the influence of cooperating APC and regulatory cells, would in this setting be required to permit the development of an effective protective response. In contrast, when the numbers of Ag-experienced cells are high, either during the early phases of an active response or in a pathologic condition of chronic tissue inflammation, Ag-independent recruitment of T cells to peripheral tissues and Ag-independent cell activation in these tissues may become clinically relevant, leading to the expression of pathologic inflammatory responses after exposure to otherwise innocuous inflammatory stimuli.
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