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IL-9-Deficient Mice Establish Fundamental Roles for IL-9 in Pulmonary Mastocytosis and Goblet Cell Hyperplasia but Not T Cell Development

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Summary

Interleukin-9 is a cytokine produced by Th2 cells and is a candidate gene for asthma and atopy. We have generated IL-9-deficient mice to delineate the specific roles of IL-9 in Th2 responses. Using a pulmonary granuloma model, we have demonstrated a distinct requirement for IL-9 in the rapid and robust generation of pulmonary goblet cell hyperplasia and mastocytosis in response to lung challenge. In contrast, eosinophilia and granuloma formation were not affected. IL-9 was not required for T cell development or differentiation, the generation of naive or antigen-driven antibody responses, or the expulsion of the intestinal parasitic nematode *Nippostrongylus brasiliensis*. Thus, deletion of IL-9 manifests as a highly defined phenotype in Th2 responses modulating mucus production and mast cell proliferation.

Introduction

Immune responses associated with atopy and helminth infection are distinguished by the generation of T helper 2 cells with their characteristic expression of cytokines such as IL-4, IL-5, IL-9, and IL-13 (Finkelman et al., 1997; Wills-Karp, 1999). Consequently, considerable interest has focused on the effector functions elicited by these cytokines, including IgE secretion, eosinophilia, and mucus production (Sanderson, 1992; Foster et al., 1996; Cohn et al., 1997; Nicolaides et al., 1997; Emson et al., 1998; McKenzie et al., 1998a, 1999). The genes encoding IL-4, IL-5, IL-9, and IL-13 are closely linked on human chromosome 5 and map to a cytokine gene cluster that also includes IL-3 and granulocyte-macrophage-colony stimulating factor (GM-CSF) (McKenzie et al., 1993; Frazer et al., 1997). These genes map to the syntenic region of mouse chromosome 11, with the exception of the IL-9 gene, which maps to mouse chromosome 13 (Mock et al., 1990).

IL-9 was first identified as a mouse T cell growth factor (Van Snick et al., 1989), but using in vitro assay systems it has also been demonstrated to modulate B cell maturation (Petit-Frere et al., 1993; Vink et al., 1999) and to promote proliferation and differentiation of mast cells (Renauld et al., 1990) and hematopoietic progenitors (Bourette et al., 1992). Recently, IL-9 has been proposed as a candidate gene for asthma based on the correlation of mapping studies carried out in mice and on human populations (Marsh et al., 1994; Nicolaides et al., 1997) and its expression profile (Shimbara et al., 2000). However, the specific role played by IL-9 in this process is unclear. It is also noteworthy that IL-9 signaling is initiated from a receptor complex that includes the IL-9 receptor (IL-9R) chain (Renauld et al., 1992) and the cytokine receptor common γ-chain (γ_c), which is found to be mutated in cases of human X-SCID. Thus, a deficit in IL-9 signaling may contribute to this syndrome (Di Santo et al., 1995).

Overexpression of IL-9 in transgenic mice has highlighted that dysregulated expression of this cytokine induces profound perturbations in multiple hematopoietic cell lineages resulting in a diverse phenotype. Thus, ubiquitous expression of an IL-9 transgene has been shown to associate with lymphomagenesis, enhanced immunoglobulin expression, expansion of B-1 lymphocyte subsets (Renauld et al., 1994; Vink et al., 1999), mastocytosis, and parasitic worm expulsion (Faulkner et al., 1997, 1998). Furthermore, expression of IL-9 transgenes under the control of a lung-specific promoter led to severe airway inflammation with infiltration of eosinophils and lymphocytes, mast cell hyperplasia, and increased subepithelial collagen deposition (Temann et al., 1998). While these data demonstrate the potent nature of the response to IL-9, their complexity makes interpretation difficult.

We have used gene targeting to generate a novel mouse line in which we have disrupted the expression of IL-9. These mice have been analyzed to determine potential roles for IL-9 in naive animals and in response to antigenic challenge and nematode infection. Significantly, disruption of IL-9 expression did not have a multifocal effect, as seen in the IL-9 overproducing transgenics. Instead, our data show that IL-9 is critical for the rapid induction of pulmonary goblet cell hyperplasia and mastocytosis following lung challenge.

Results

Generation of IL-9-Deficient Mice

The targeting vector comprised a 5′ arm of homology and a 3′ arm of homology positioned either side of the neomycin resistance cassette (Figure 1A). The resulting homologous recombination event occurred at a ratio of 1 in 175 neomycin resistant clones. Genotyping of wild-type (*IL-9*$^{+/+}$), heterozygous (*IL-9*$^{+/-}$), and homozygous null (*IL-9*$^{-/-}$) mice is shown in Figure 1B. IL-9-deficient mice were healthy and displayed no overt phenotypic abnormalities. Analysis of the IL-9-deficient mice failed to detect IL-9 RNA transcripts from activated lymphocytes using reverse transcriptase-polymerase chain reaction assays (Figure 1C), and enzyme-linked immunosorbent assays also failed to identify IL-9 protein in supernatants from splenocyte cultures (Figure 1D). To

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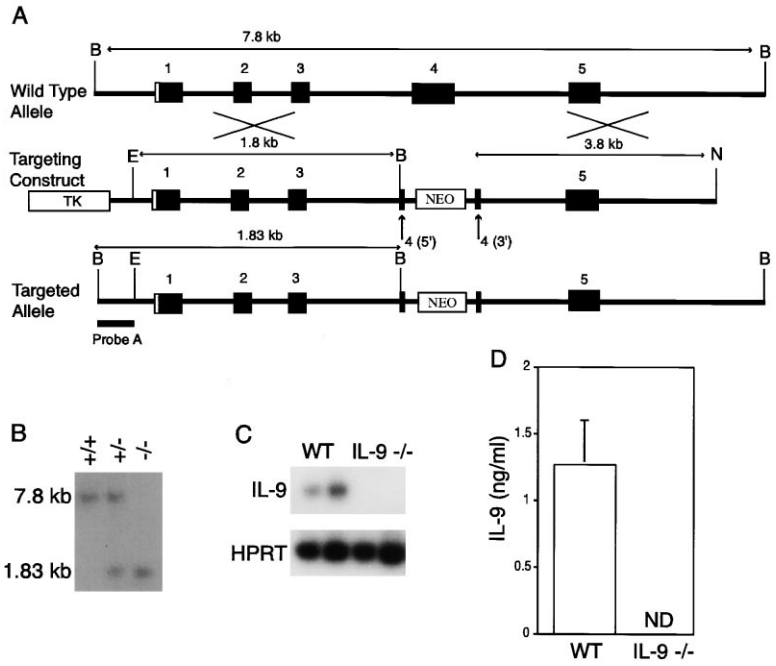

Figure 1. Inactivation of the *IL-9* Gene by Homologous Recombination

(A) The structure of the *IL-9* locus, the targeting vector, and the predicted homologous recombination event are shown. NEO, neomycin resistance cassette; TK, thymidine kinase cassette; B, BamHI; E, EcoRI; and N, NotI.

(B) Southern blotting of F_2 tail genomic DNA. The indicated probe detects a 7.8 kb BamHI fragment in the wild-type *IL-9* gene and a 1.83 kb fragment as a result of the correct homologous recombination event.

(C) RT-PCR analysis of IL-9 expression. Splenocytes were stimulated with plate-bound anti-CD3 for 48 hr, RNA was prepared, and PCR was performed. WT, wild-type.

(D) Analysis of IL-9 expression from splenocytes. Spleen cells (2.5×10^6 per ml) were stimulated with plate-bound anti-CD3 (1 μg/ml), anti-CD28 (10 μg/ml), and IL-2 (5 ng/ml) for 72 hr. Supernatants were analyzed by ELISA. Data are representative of two repeat experiments. ND, not detected; open bars, wild-types; and filled bars, *IL-9*$^{-/-}$.

determine the in vivo roles of IL-9, we have assessed the immune responses of the IL-9-deficient animals to a range of immunological challenges that normally provoke a Th2 phenotype.

Naive Cytokine Levels, Th1 and Th2 Cell Development, and Total Serum Immunoglobulin Production Are Normal in IL-9-Deficient Mice

Since in vitro assays have demonstrated a role for IL-9 in T cell activation, we wished to address whether the disruption of IL-9 expression in vivo would alter the levels of T cell cytokine production. Polyclonal stimulation of mesenteric lymph node cells from naive animals with anti-CD3 demonstrated that IL-5, IL-10, and IFN-γ production was not altered by the absence of IL-9 (Figure 2A). Similarly, when lymph node cells were cultured in vitro under conditions that promote differentiation of Th1 or Th2 cells, no significant differences were observed in the generation of these lineages in the IL-9-deficient or wild-type mice (Figure 2B). In keeping with the unaltered expression of cytokines, the total serum immunoglobulin isotype responses detected in the IL-9-deficient mice were comparable to those in the wild-type controls (Figure 2C). Furthermore, FACS analysis of cell suspensions prepared from lymph node, spleen, bone marrow, peritoneum, and thymus, from naive IL-9-deficient and wild-type mice, demonstrated no differences in the expression of the following cell surface markers: CD4, CD8, T cell receptor, CD3, CD5, CD25, CD45, Gr-1 on thymocytes; CD4, CD8, T cell receptor, CD3, CD5, CD14, CD23, CD45, Gr-1, CD11b, TER119 on mesenteric lymph node cells or splenocytes; CD3, CD5, CD14, CD23, CD24, CD43, CD45, Gr-1, CD11b, TER119, BP1, c-kit on bone marrow; CD5, CD45, CD11b, c-kit, Gr-1 on peritoneal lavage (data not shown).

Antigen-Specific Cytokine and Antibody Responses Are Normal in IL-9-Deficient Mice

We also assessed whether the absence of IL-9 would alter the induction of antigen-specific systemic immune responses following immunization with antigen. IL-9-deficient and wild-type mice were challenged with ovalbumin complexed with the adjuvant alum and their cytokine and immunoglobulin responses analyzed. Mesenteric lymph node cells from the immunized mice were restimulated in vitro using either anti-CD3 or antigen and their cytokine production determined. Polyclonal and antigen-driven cytokine responses from immunized IL-9-deficient mice were comparable to those produced by the wild-type control animals (Figure 3A). Similarly, antigen-specific immunoglobulin responses were also unaltered by the absence of IL-9 expression (Figure 3B). Thus, despite reports that IL-9 can alter the in vitro differentiation of B cells and enhance total immunoglobulin expression, we have been unable to demonstrate a significant role for IL-9 in this process in vivo following IL-9 gene disruption. Our data indicate that in the absence of IL-9 compensatory pathways exist for the normal development of immunoglobulin responses.

Goblet Cell Hyperplasia and Mastocytosis Is Severely Impaired in IL-9-Deficient Animals following the Induction of Synchronous Pulmonary Granuloma Formation

To determine the in vivo contribution of IL-9 in a Th2 cytokine-mediated inflammatory response, we employed a model system in which synchronous pulmonary granuloma formation is induced around *Schistosoma mansoni* eggs (Warren and Domingo, 1970). In this model, a cellular granulomatous response develops around parasite eggs that lodge in the lungs following their intravenous injection into mice. This inflammatory

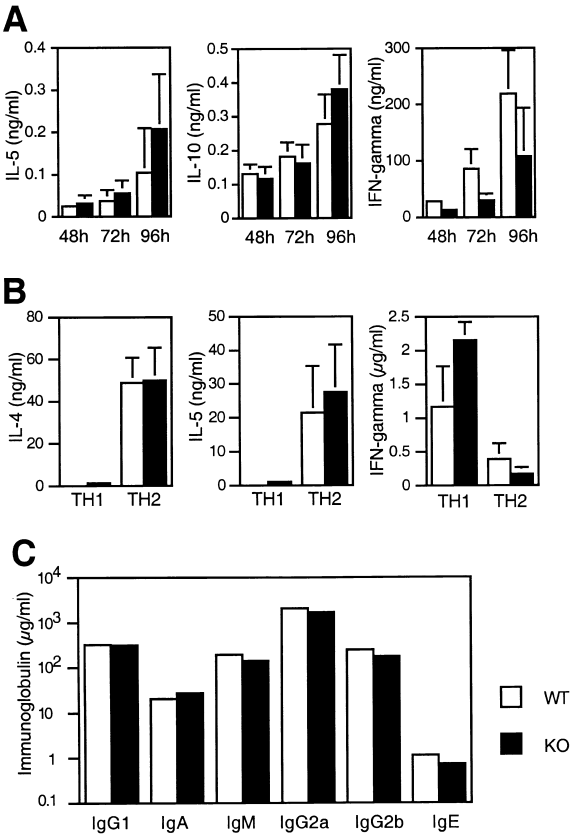

Figure 2. Analysis of Naive Immune Responses

(A) Polyclonal activation of mesenteric lymph node (MLN) cells. MLN cells were stimulated with plate-bound anti-CD3 for the times shown. Data represent means plus SD of triplicate wells. IL-4 was below the levels of detection.

(B) Cytokine production following in vitro Th cell differentiation. Data represent means plus SD of triplicate wells.

(C) Total serum immunoglobulin isotype production. Sera from three animals were pooled. Data represent means plus SD of triplicate wells. All data are representative of two repeat experiments. Open bars, wild-types; and filled bars, *IL-9*$^{-/-}$.

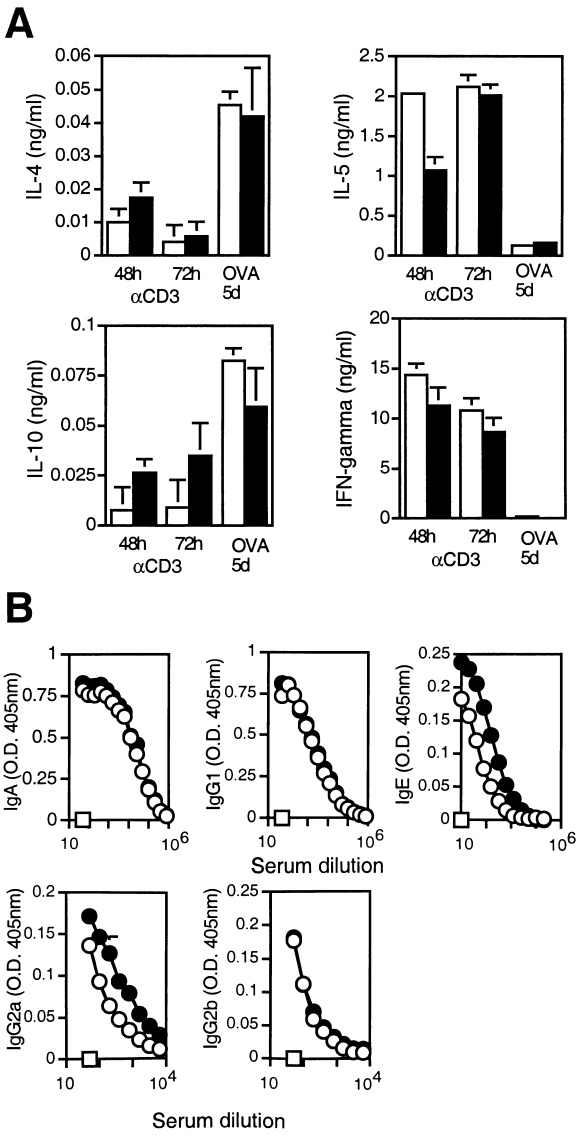

Figure 3. Antigen-Specific Response of IL-9-Deficient and Wild-Type Mice to Ovalbumin Immunization

(A) Cytokine responses. Open bars, wild-types; and filled bars, *IL-9*$^{-/-}$.

(B) Immunoglobulin responses. Cohorts of four to five animals were immunized intraperitoneally with ovalbumin. Serum samples were assayed by ELISA for immunoglobulin isotypes. Data are presented as means plus SD. Representative data from two repeat experiments are shown. Open circles, immunized wild-types; filled circles, immunized *IL-9*$^{-/-}$; open square, naive wild-types; and filled square, naive *IL-9*$^{-/-}$.

response is characterized by the high-level expression of Th2 cytokines (Wynn et al., 1993), eosinophil infiltration (McKenzie et al., 1999), and goblet cell hyperplasia. This model also has the advantage of generating an inflammatory response that can be quantified following either a primary or secondary exposure to antigen (McKenzie et al., 1999; Townsend et al., 2000).

Significantly, following primary lung challenge, histological analysis demonstrated that in contrast to the wild-type controls, the IL-9-deficient animals failed to develop the goblet cell hyperplasia normally found along the airway epithelia (Figure 4A). Quantification of the goblet cells demonstrated that 10-fold fewer goblet cells were induced in the absence of IL-9 expression (Figure 4B). IL-9-deficient mice also failed to develop the mastocytosis evident in the lungs of the immunized wild-type mice. Enumeration of toluidine stained mast cells indicated that lungs from IL-9-deficient mice contained 8-fold fewer mast cells than the wild-type controls (Figure 4C). Furthermore, enumeration of pulmonary intraepithelial mast cells using chloroacetate esterase staining also demonstrated fewer positive cells in the IL-9-deficient mice (wild-type, 6.5 ± SE 0.329; *IL-9*$^{-/-}$, 2.672 ± SE 0.321 [P = 0.0004 by Student's *t*-test], defined as positive cells per 40 field of view). In contrast, we found no evidence for a decrease in the numbers of bone marrow mast cells in the IL-9-deficient animals, as determined by c-kit staining (wild-type, 13.4–15.01%; *IL-9*$^{-/-}$, 11.96–15.89%; defined as percentage c-kit positive cells of total bone marrow cells gating out erythrocytes). Moreover, in vitro mast cell cultures generated from the

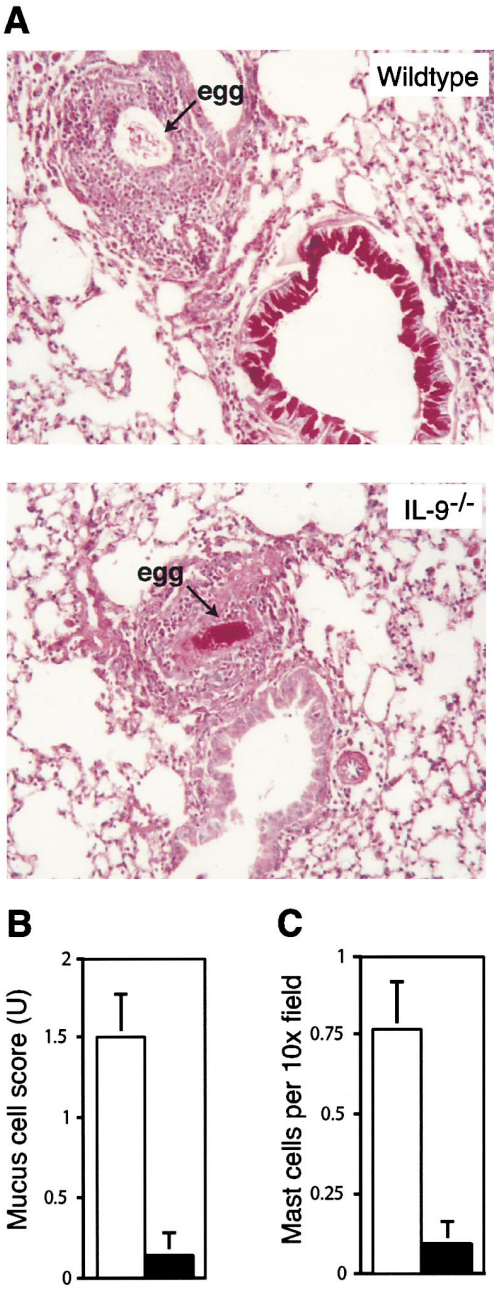

Figure 4. Goblet Cell Hyperplasia and Mastocytosis during the Primary Pulmonary Inflammatory Response

(A) Histological analysis of goblet cells in wild-type and *IL-9*$^{-/-}$ lung. Lung sections were stained with PAS. Wild-type image demonstrates epithelial goblet cell hyperplasia (stained red) around an airway adjacent to cellular infiltrate induced by an entrapped egg. Despite similar cellular infiltration, goblet cell hyperplasia is not evident in the section from the IL-9-deficient sample. Magnification, ×20.

(B) Determination of goblet cell number. Enumeration of PAS positive goblet cell numbers to give mucus cell score. Data are presented as means plus SE.

(C) Toluidine blue stained mast cell numbers from wild-type and IL-9-deficient mice. Data are presented as means plus SE.

All data are representative of three repeat experiments using four to six mice per group. Open bars, wild-types; and filled bars, *IL-9*$^{-/-}$.

bone marrow cells of wild-type and IL-9-deficient mice, cultured in the presence of IL-3 and stem cell factor (McKenzie et al., 1998b), also displayed equivalent mast cell development (data not shown). These data indicate that the deficit in mast cell expansion observed in the IL-9-deficient lung tissue does not result from the inefficient generation of mast cell precursors but probably reflects a defect in the proliferation of differentiated cells.

To determine whether the differences observed in the goblet cell hyperplasia were simply due to a decrease in pulmonary inflammation, we analyzed the composition of the granulomas and lung tissue. We observed that both the IL-9-deficient and wild-type animals developed granulomas of equivalent size and cellularity (Figures 5A and 5B), with eosinophils constituting ~30% of the granuloma (Figure 5C). Furthermore, analysis of lung tissue and bronchoalveolar lavage (BAL) showed no difference in the inflammatory response. Pulmonary fibrosis as assessed by collagen deposition was equivalent in the two mouse lines (Figure 5D), as were the proportions of eosinophils, neutrophils, macrophages, and lymphocytes in the BAL fluid (Figure 5E). In addition, we also analyzed the levels of the cellular adhesion molecules ICAM-1 (CD54) and VCAM-1 (CD106), which are normally associated with inflammatory processes (Bevilacqua, 1993). Histological examination of lung tissue from immunized IL-9-deficient and wild-type animals demonstrated equivalent staining for ICAM-1 and VCAM-1 (Figure 5F) as determined using the arbitrary scoring system outlined in Experimental Procedures. Since IL-4, IL-5, and IL-13 have been shown to play important roles in the development of pulmonary Th2 responses, we assessed the expression of these cytokines from cultures prepared from the draining mediastinal lymph nodes of wild-type and IL-9-deficient mice following primary immunization. Significantly, we found equivalent antigen-induced expression of IL-4, IL-5, and IL-13 by the wild-type and IL-9-deficient mice (Figure 5G). Thus, the observed reduction in goblet cell and mast cell numbers in the IL-9-deficient mice occurs independently of these cytokines, indicating that IL-9 is a key regulator of these processes. Moreover, these data also indicate that the induction of IL-4, IL-5, and IL-13 is not regulated by IL-9.

Previous studies using cytokine-deficient mice have demonstrated that IL-4 and IL-5 do not play an essential role in Th2 cell driven pulmonary mucus production (Cohn et al., 1997, 1999). However, IL-4Rα, a shared component of the IL-13 receptor complex, is critical for this process (Cohn et al., 1999), implying a role for IL-13 in the regulation of goblet cells. IL-13 has also been reported to act in the generation of goblet cell responses (Grunig et al., 1998; McKenzie et al., 1998a; Wills-Karp et al., 1998). In order to investigate the interaction of IL-13 and IL-9 in the regulation of goblet cell hyperplasia, we have assessed primary granuloma formation in IL-13-deficient mice. In marked contrast to the specific reduction in goblet cell responses observed in IL-9-deficient animals, IL-13-deficient animals developed a global impairment in their Th2 response with a concomitant reduction in granuloma volume (Figure 6A), 3-fold fewer goblet cells (Figure 6B), a reduction in eosinophilia (Figure 6C) and fibrosis (Figure 6D), and reduced levels of

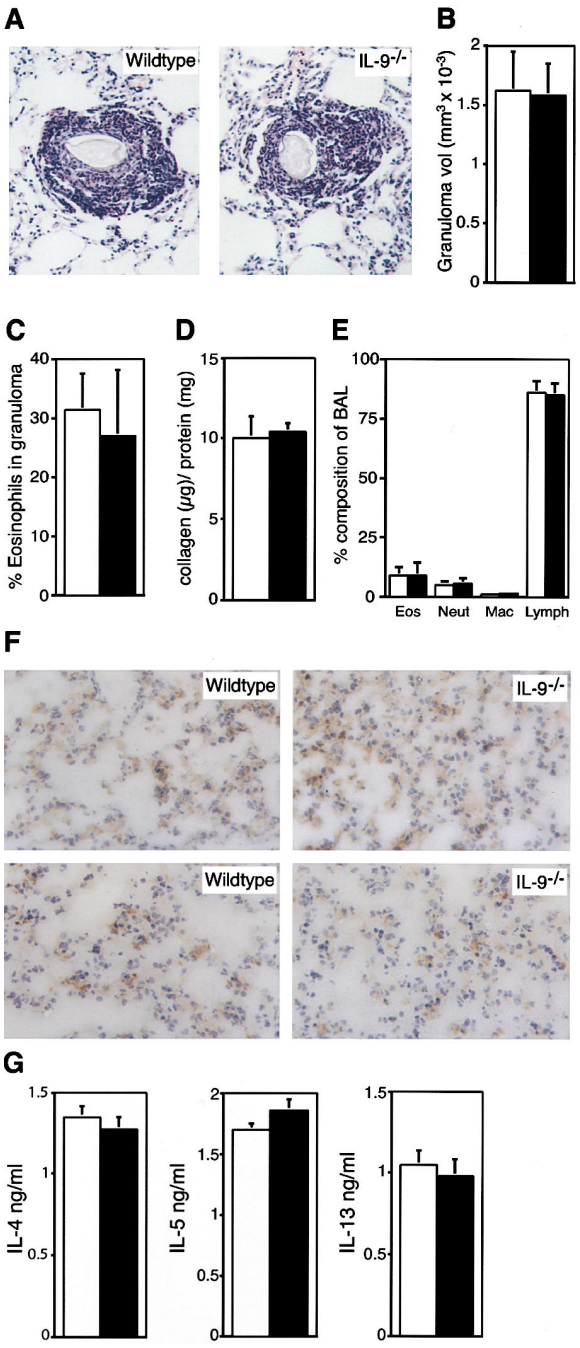

Figure 5. Granuloma Formation and Eosinophilia during the Primary Pulmonary Inflammatory Response

(A) Morphological analysis of granuloma formation in wild-type and *IL-9*$^{-/-}$ mice. Lung sections were stained with haematoxylin and eosin. Magnification, ×20.

(B) Determination of granuloma volumes in immunized mice. Lung sections were stained with haematoxylin and eosin, and at least 100 individual granulomas were measured per group. Data are presented as means less egg volumes plus SE.

(C) Determination of granuloma eosinophils. The percentage eosinophils per granuloma was determined from lung sections, and at least 100 individual granulomas were examined per group. Data are presented as means plus SE.

(D) Determination of pulmonary fibrosis. Pulmonary collagen was quantified by differential staining of sections and expressed as microgram of collagen per milligram of protein. Data are presented as means plus SE.

(E) Determination of bronchoalveolar lavage (BAL) cell composition. Cytocentrifuge preparations of BAL cells were stained with Giemsa and differential counts performed. Eos, eosinophils; neut, neutrophils; mac, macrophages; and lymph, lymphocytes. Data are presented as means plus SE.

(F) Histological analysis of ICAM-1 (top panels) and VCAM-1 (bottom panels) expression in the alveolar parenchyma of primary challenged wild-type and *IL-9*$^{-/-}$ mice. Magnification, ×40. Expression of ICAM-1 and VCAM-1 (stained brown) was evaluated double-blind using an arbitrary scoring system.

(G) Cytokine responses from activated lymph node cells. Draining mediastinal lymph node cells were cultured for 4 days with soluble egg antigen. Supernatants were analyzed by cytokine ELISA. Data are presented as means plus SE.

Cohorts of four to six mice were injected intravenously with 5000 schistosome eggs to induce synchronous pulmonary granuloma. Mice were killed 14 days later. Data are representative of two repeat experiments. Open bars, wild-type; and filled bars, *IL-9*$^{-/-}$.

IL-4, IL-5, and to a lesser extent IL-9 (Figure 6E). Thus, both IL-9 and IL-13 can regulate goblet cell hyperplasia. Critically, since in the IL-9-deficient mice IL-13 was expressed normally but could not compensate for the lack of IL-9 in the primary response, this highlights an important primary role for IL-9 in the initiation of the goblet cell response. Thus, the primary pulmonary granulomatous model demonstrates that although mice deficient in IL-9 are still capable of mounting an eosinophil-rich Th2-mediated inflammatory response, they fail to generate the normal goblet cell hyperplasia and mastocytosis associated with primary lung challenge. It is noteworthy that following secondary challenge the pulmonary granulomatous response produces an equivalent induction of goblet and mast cells even in the absence of IL-9 expression (data not shown). These data demonstrate that the role of IL-9 in this response is an early event and that further compensatory factors can modulate the goblet cell response following priming.

Responses to the Parasitic Nematode *N. brasiliensis* Are Normal in IL-9-Deficient Mice

Immunological responses to gastrointestinal parasitic worm infections are also characterized by the expression of Th2 cytokines (Finkelman et al., 1997). A potential role for IL-9 in the expulsion of parasitic nematodes has been suggested by the expression profile of this cytokine during infections and by the extremely rapid expulsion of parasites by IL-9-overexpressing transgenic mice (Faulkner et al., 1997, 1998). Using *N. brasiliensis* as a model, we have infected the IL-9-deficient mice and compared worm expulsion with that of wild-type mice. The wild-type and IL-9-deficient mice displayed very similar worm burdens at day 5 postinfection (p.i.), and both groups had expelled their worms completely by day 10 p.i. (Figure 7A). However, we found reproducibly fewer goblet cells in the intestines of the IL-9-deficient mice than the wild-types (Figure 7B). We also assessed intestinal mast cells associated with *N. brasiliensis* infection. Intraepithelial mast cells were detected on sections of jejunum from infected mice, by staining with toluidine blue or using cytochemical staining of sections to detect chloroacetate esterase activity

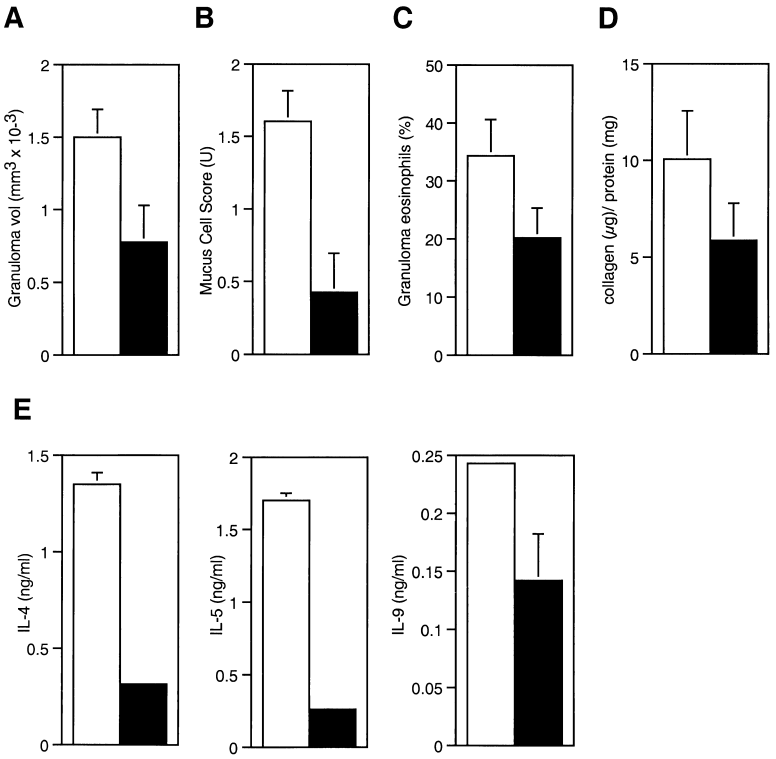

Figure 6. Analysis of Primary Pulmonary Inflammatory Response in *IL-13*$^{-/-}$ Mice

(A) Determination of granuloma volumes in immunized wild-type and *IL-13*$^{-/-}$ mice. Lung sections were stained with haematoxylin and eosin, and at least 100 individual granulomas were measured per group. Data are presented as means less egg volumes plus SD.

(B) Determination of goblet cell number in wild-type and *IL-13*$^{-/-}$ lung. Lung sections were stained with PAS, and goblet cell numbers were counted to give mucus cell score.

(C) Determination of granuloma eosinophils. The percentage eosinophils per granuloma was determined from lung sections, and at least 100 individual granulomas were examined per group. Data are presented as means plus SD.

(D) Determination of pulmonary fibrosis. Pulmonary collagen was quantified by differential staining of sections and expressed as microgram of collagen per milligram of protein. Data are presented as means plus SD.

(E) Cytokine responses from activated lymph node cells. Draining mediastinal lymph node cells were cultured for 4 days with soluble schistosome egg antigen. Supernatants were assayed for cytokines by ELISA. Data are presented as means plus SD.

Cohorts of four mice were injected intravenously with 5000 schistosome eggs to induce synchronous pulmonary granuloma as described above. Data are representative of two repeat experiments. Open bars, wild-types; filled bars, *IL-13*$^{-/-}$.

present in all mouse mast cells (Friend et al., 1996). Although we observed a general trend of fewer intraepithelial mast cells in IL-9 deficient mice compared to wild-type mice, the differences were not statistically significant. For example, at day 10 postinfection wild type mice ($n = 5$) had 4.42 ± 0.57 esterase positive mast cells per villus crypt unit (mean $\pm$ SE) whereas IL-9-deficient mice ($n = 4$) had 2.94 ± 0.52 mast cells per villus crypt ($p < 0.11$ by Student's *t*-test). Previously, it has been shown that mice overexpressing IL-9 have marked mast cell infiltration of the muscularis mucosa (Godfraind et al., 1998). To more accurately determine the full extent of intestinal mastocytosis in nematode-infected mice, we measured the levels of mast cell proteases (mMCP-1) in homogenates of jejunum from infected mice. Throughout the course of infection with *N. brasiliensis*, mMCP-1 activity in the jejunum was lower in IL-9 deficient mice compared to wild type mice (Figure 7C). It is appropriate to highlight that in separate nematode infection studies, although we have consistently observed reduced intestinal mastocytosis in IL-9 deficient mice, based on two different mast cell histological stains and detection of mast cell protease activity, these differences have not been statistically significant. Antigen-specific antibody responses following infection were also comparable between the two groups (data not shown). Polyclonal and antigen-specific cytokine production from the mesenteric lymph node cells of the IL-9-deficient and wild-type mice indicate comparable cytokine responses at day 5 p.i. (Figure 7D) but suggest that by day 10 p.i. the IL-9-deficient mice are making more antigen-specific Th2 cytokines (Figure 7E), possibly indicating a requirement for compensation in these animals.

Discussion

In order to investigate the in vivo roles of IL-9, we have generated a novel line of IL-9-deficient mice. The immune functions of these animals have been evaluated both in naive mice and in animals following immunization or infection. These studies have highlighted an important role for IL-9 in the rapid and robust generation of pulmonary goblet cell hyperplasia and mastocytosis. However, our studies have failed to show a significant role for IL-9 in T cell development or differentiation, the generation of naive or antigen-driven antibody responses, or the expulsion of the intestinal parasitic nematode *N. brasiliensis*.

Despite reports that IL-9 can act as a growth factor for certain T cell clones (Van Snick et al., 1989) and the observed development of thymic lymphomas in IL-9 transgenic mice (Renauld et al., 1994), we were unable to identify changes in the T cell subpopulations of the thymus or peripheral lymphoid tissues from the IL-9-deficient mice. Thus, if IL-9 does play a role in T cell development, alternative pathways must function in its absence. This represents an important finding, since IL-9 binds to a receptor complex that includes the IL-9R and γ_c, and it has been suggested that IL-9 may represent an additional signal that is missing in human patients suffering from X-SCID, which is caused by muta-

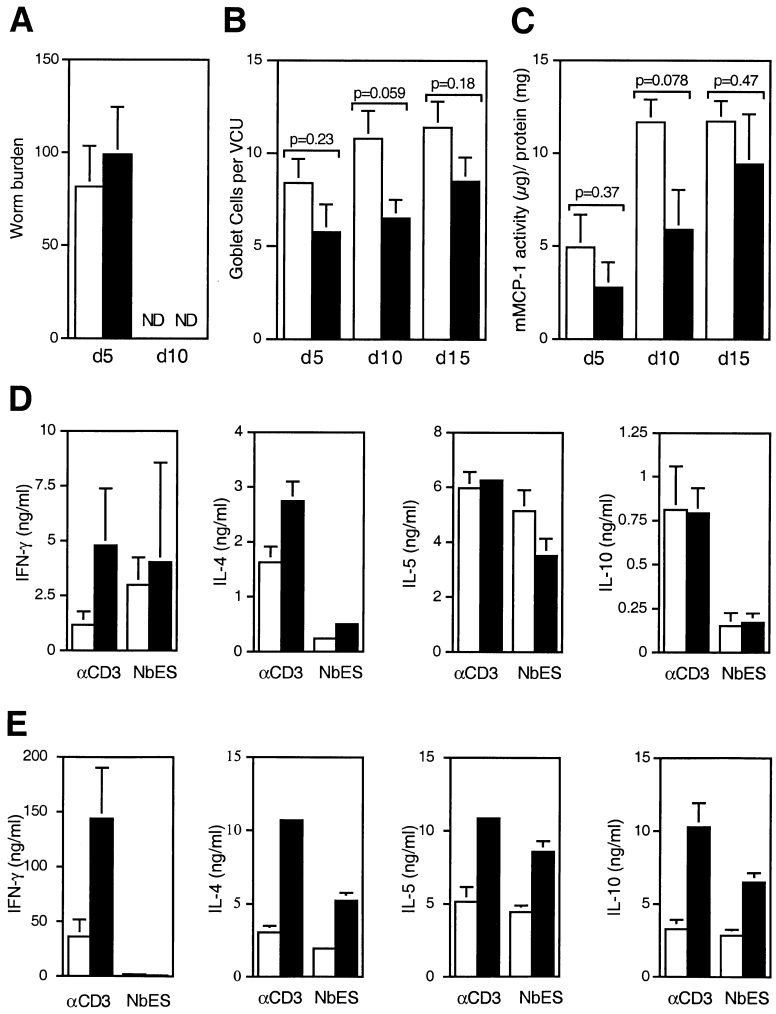

Figure 7. Infection with *Nippostronglyus brasiliensis*

(A) Infected mice were sacrificed at the times indicated to obtain intestinal worm counts. Data are representative of two repeat experiments. Open bars, wild-types; filled bars, *IL-9*$^{-/-}$; and ND, not detected.

(B) Determination of goblet cell number. Enumeration of PAS positive goblet cells per villus crypt unit (VCU). Data are presented as means plus SE with p value (Student's *t*-test).

(C) Mouse mast cell protease-1 (mMCP-1) activity was measured from jejunal homogenates. Data are presented as means plus SE with p value (Student's *t*-test).

(D) Cytokines at day 5 postinfection. Mesenteric lymph node cells from wild-type and *IL-9*$^{-/-}$ animals following infection with *N. brasiliensis* were stimulated with anti-CD3 or *N. brasiliensis* antigen (NbES). Supernatants were analyzed by cytokine ELISA. Data are presented as means plus SD.

(E) Cytokines at day 10 postinfection. Mesenteric lymph node cells were stimulated as described for day 5. Data are presented as means plus SD.

Cohorts of five mice were infected with 500 viable third stage *N. brasiliensis* larvae. Data are representative of two repeat experiments. Open bars, wild-types; and filled bars, *IL-9*$^{-/-}$.

tions in the γ_c gene (Di Santo et al., 1995). However, it will be interesting to determine whether an additive effect of IL-9 in T cell development may be found by intercrossing the IL-9-deficient mice with other mouse lines deficient in the expression of cytokines that utilize γ_c, for example IL-2, IL-4, IL-7, and IL-15.

We have also found that immunoglobulin responses are unaltered by the absence of IL-9. These data differ from those reported for the IL-9 transgenic mice that display a global increase in all immunoglobulin isotypes (Vink et al., 1999). While it is not clear why this should be the case, it seems likely that aberrant overexpression of IL-9 induces indirect upregulation of B cell function, which does not represent a critical role for IL-9. To date, we have also failed to demonstrate any change in the number of peritoneal B-1 cells in the IL-9-deficient mice. These data also contrast with the findings from IL-9 transgenic mice that display an expansion of a B-1 lymphocyte population that is independent of IL-5 regulation (Vink et al., 1999). It seems likely that the IL-9-responsive population of B-1 cells represents only a small proportion of cells in naive mice, making changes in this subset difficult to identify and only following extensive overexpression of IL-9 do they become apparent. Further analysis will be necessary to examine whether such B-1 cell populations become evident following various antigenic challenges or when IL-9 deletion is performed in combination with the disruption of IL-5.

Using the pulmonary granuloma model, we have been able to study the onset of Th2 responses during both primary and secondary challenges in order to identify obligate roles for IL-9 in these immune responses. This approach has proved highly illuminating, identifying that IL-9 is critically important for the rapid onset of goblet cell hyperplasia and the upregulation of mast cells but not for eosinophilia, fibrosis, or lymphocyte function.

Th2 cell-mediated mucus production represents an important response for protecting mucosal surfaces. Although mucus production acts positively in the protection of mucosal surfaces from pathogens and by assisting in parasite expulsion (Nawa et al., 1994), it may also be detrimental by contributing to airway obstruction during pulmonary inflammatory responses (Rogers, 1994). The regulation of goblet cell mucus production by cytokines has been attributed to IL-4, IL-9, and IL-13. While IL-4 is presumed to be necessary for the generation of IL-9 and IL-13 producing Th2 cells (Cohn et al., 1999), IL-9 and IL-13 are believed to directly regulate goblet cell production and function (Grunig et al., 1998; McKen-

zie et al., 1998a; Wills-Karp et al., 1998; Cohn et al., 1999; Louahed et al., 2000). Our data from IL-9-deficient mice now demonstrate that IL-9 is a critical factor for initiating the primary goblet cell response following primary pulmonary challenge. Significantly, this regulation of goblet cell hyperplasia is independent of IL-13, IL-4, and IL-5. Moreover, IL-9-deficient mice developed otherwise normal pulmonary Th2 inflammatory responses with characteristic eosinophilia, ICAM-1 and VCAM-1 expression, fibrosis, and BAL cell composition, indicating that the goblet cell defect is not due to a diminution of the inflammatory response but is specific to the deficit in IL-9 expression.

There is obvious complexity to the interrelated roles of IL-9 and IL-13 in the regulation of goblet cell production. Clearly, IL-13 expression in the primary challenge is normal yet fails to compensate for the absence of IL-9. Therefore, IL-9 must act independently of IL-13 in initiating this response. However, it is apparent that IL-13 is also an important factor in the regulation of goblet cell responses. It is possible that IL-13 may perform a role as a goblet cell-enhancing factor acting independently of IL-9 and with slower kinetics or that it is necessary for developing the Th2 response and increasing IL-9 expression. Our experiments using IL-13-deficient mice indicate that in the absence of IL-13, goblet cell hyperplasia is also impaired though to a lesser degree than in the IL-9-deficient mice. In contrast to the IL-9-deficient mice, the IL-13-deficient mice also develop a global downregulation of the Th2 response with smaller granulomas, a reduction in eosinophilia and fibrosis, and reduced levels of IL-4, IL-5, and IL-9. The normal levels of goblet cells induced following secondary challenge of the IL-9-deficient mice indicate that alternative IL-9-independent mechanisms can compensate for the absence of IL-9 following sensitization. Future studies will address the interrelated role of IL-13 in this process. Our results demonstrating a key role for IL-9 in mucus production are supported by a study of allergen-challenged dogs in which IL-9, but not IL-5 or IL-13, was found to be the only cytokine that correlated with mucus production. The authors further showed that epithelial cells express IL-9R and that tracheal administration of IL-9 enhanced goblet cell number (Longphre et al., 1999). Furthermore, Louahed et al. (2000) have demonstrated that IL-9 and IL-13 can independently regulate mucin gene expression following treatment of mice or culture of human pulmonary epithelial cells.

Elevated numbers of mast cells are characteristic of Th2 responses, being produced in response to helminth infection, and are thought to contribute to the pathology of pulmonary disease by releasing vasoactive mediators that exacerbate inflammation following IgE-cross-linking (Costa et al., 1997). Both in vitro and in vivo studies have indicated that IL-9 plays a contributory role in the generation of mast cells (Renauld et al., 1990; Faulkner et al., 1997; Godfraind et al., 1998; Temann et al., 1998). Our study supports these findings, indicating that in the absence of IL-9 the normal increase in mast cell number following lung challenge is severely curtailed. Thus, IL-9 appears to be important for the rapid generation of mast cells following antigen challenge. Since we observed no deficit in the generation of bone marrow mast cells or the outgrowth of mast cells from bone marrow cell cultures, IL-9 does not appear to effect mast cell progenitor production but apparently enhances mast cell expansion in the lung tissue. These data contrast with those reported for IL-3-deficient mice in which impaired mast cell outgrowth results from a defect in mast cell progenitor development (Lantz et al., 1998). Interestingly, we also observed that prolonged in vivo exposure to antigen, in the form of a secondary immunization, overrides the dependence on IL-9, and other factors can then compensate for the role of IL-9 in mastocytosis. Certainly stem cell factor, IL-3, and IL-4 are all capable of inducing mast cell outgrowth (Galli and Hammel, 1994).

Despite our observations in the lung demonstrating that IL-9 plays a significant role in modulating the immune response to antigen, we have been unable to determine an essential role for IL-9 in the expulsion of the parasitic nematode worm *N. brasiliensis*. Although we observed fewer goblet cells in the IL-9-deficient mice, parasite expulsion was not delayed by the absence of IL-9. However, we did observe an increase in IL-4 and IL-5 production by restimulated cells from the mesenteric lymph node of IL-9-deficient mice. This may indicate that the disruption of IL-9 expression alters the balance of the normal response necessitating an increase in the expression of other Th2 cytokines, which then compensate for the loss of IL-9. Although studies have shown that IL-9 transgenic mice expel nematode parasite infections more efficiently than wild-type animals, the physiological significance of these findings is unclear since naive IL-9 transgenic mice display a severely altered immune system. However, it is possible that analysis of other parasitic worm models, which vary in their modes of colonisation, may uncover a mechanism that is hidden in the *N. brasiliensis* model.

In conclusion, our data have highlighted an important role for IL-9 in the rapid onset of goblet cell hyperplasia and mastocytosis in a pulmonary Th2 response. These results may have important consequences in the fields of asthma and allergy, and these mice should prove an important resource for the evaluation of IL-9 function in models of airway hyperresponsiveness. The transient nature of the observed defect in goblet cell hyperplasia and mastocytosis also emphasises the complex nature of the cytokine response and its capacity for compensation. Future studies will attempt to address the interrelated roles of the Th2 cytokines using intercrossing of cytokine-deficient and cytokine-transgenic mice to probe the causal relationships involved in these responses.

Experimental Procedures

Targeted Disruption of the Mouse *IL-9* Gene in Embryonic Stem Cells

The replacement vector was constructed to insert the neomycin resistance gene into exon 4 of the *IL-9* gene deleting the nucleotides encoding amino acids 54–59. Stop codons in all three frames were inserted 5′ of the selectable marker. The targeting vector consisted of 1.8 kb of the *IL-9* gene providing the 5′ arm of homology and 3.8 kb comprising the 3′ homology arm. The targeting vector was linearized and electroporated into E14.1 embryonic stem (ES) cells (McKenzie et al., 1998b). Of 700 G418 resistant clones screened by Southern analysis, using an external flanking probe, four were found to be targeted correctly. Hybridization with a probe to the neomycin sequence confirmed the predicted size of the targeted fragment

and that only a single integration had occurred. The targeted ES cell clone was microinjected into 3.5 day C57BL/6 blastocysts to generate chimeras. These mice were mated with C57BL/6 mice and transmitted the ES cell genotype through the germline. Mice homozygous for the disrupted *IL-9* gene were obtained by interbreeding the heterozygotes. IL-13-deficient mice were those generated as described previously (McKenzie et al., 1998a). The IL-9 gene-targeted, IL-13 gene targeted, and wild-type animals used in the experiments reported below were maintained on a 129 × C57BL/6 (F_2) background in a specific pathogen free environment.

Stimulation of Naive Lymphocytes and RNA Preparation

Mesenteric lymph node cells or splenocytes from three animals were pooled and cultured at 2.5×10^6 cells per ml on plates coated with anti-CD3. Total RNA was prepared using RNAzolB. RT-PCR primers for IL-9 were 5′-AGACACCATGGACCTTATTTAAAATCT GAAGG-3′ and 5′-GGACACGTTATGTTCTTTAGG-3′. The internal oligonucleotide used for hybridization was 5′-CTGCTTGTGTCT CTCCGTCCC-3′. HPRT primers and conditions were as described in Reiner et al. (1993).

In Vitro T Helper Cell Differentiation Assays

Mesenteric lymph node cells were cultured on anti-CD3ε antibody-coated plates (1 μg/ml of clone 2C11, Becton Dickinson) in the presence of exogenous cytokines or anti-cytokine antibody as indicated. IL-2 (10 ng/ml, R&D) was added to all cultures. Th2 cell differentiation was promoted in the presence of 100 ng/ml IL-4 (R&D) and anti-IFNγ antibody (10 μg/ml of clone XMG1.2, Becton Dickinson), while Th1 differentiation was promoted by anti-IL-4 antibody at 10 μg/ml (clone 11B11, DNAX Research Institute) and IL-12 (1 ng/ml, Genzyme, West Malling, UK). Cells were cultured for 5 days, washed, and resuspended at 1×10^6 cells per ml for 24 hr in the presence of plate-bound anti-CD3. Supernatants were analyzed by cytokine ELISA.

Pulmonary Granuloma Formation

Primary synchronous pulmonary granulomas were induced by intravenous injection of mice with *S. mansoni* eggs (Warren and Domingo, 1970). *S. mansoni* eggs were isolated from the livers of infected mice as described (Fallon et al., 1998). Fourteen days after intravenous egg injection, mice were sacrificed and the lungs were inflated with formal saline (10% formaldehyde in saline) and processed for histology. The horizontal and perpendicular diameters of the granulomas surrounding individual eggs were measured with an ocular micrometer. All sections were measured by the same individual using a blind protocol. The volume of the egg granuloma was calculated assuming a spherical shape. >100 individual granulomas were analyzed per group. Goblet cells were counted on Periodic Acid-Schiff (PAS)-stained lung sections using an arbitrary scoring system, as described (Grunig et al., 1998). PAS-stained goblet cells in airway epithelium were measured double-blind using a numerical scoring system (0: <5% goblet cells; 1: 5 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >75%) The sum of airway scores from each lung was divided by the number of airways examined, 20–50 airways per mouse, and expressed as mucus cell score in arbitrary units (U). Granuloma eosinophil infiltration was counted on Giemsa stained sections, and the numbers of eosinophils expressed as a percentage of the cell composition of the granuloma. Pulmonary collagen was quantified by differential staining of sections and expressed as microgram of collagen per milligram of protein (Fallon and Dunne, 1999). Toluidine blue stained mast cells were enumerated by counting the number of positively stained cells per ×10 field of view with 20 fields per mouse (Miller et al., 1994). Chloroacetate esterase specific staining of mast cells was performed using the Leder esterase stain (Leder, 1979), as modified by Friend et al. (1996), and the number of positively stained mast cells was counted per ×400 field of view.

Bronchoalveolar lavage (BAL) fluids were collected by cannulating the trachea and lavaging the lungs with a 1.0 ml of ice-cold PBS. The BAL cells were pelleted, washed, and counted. The numbers of eosinophils, neutrophils, macrophages and lymphocytes was determined by performing a differential count on at least 400 cells/slide of Giemsa stained cytocentrifuge preparations.

Secondary synchronous pulmonary granulomas were induced as follows. Mice were sensitised to schistosome eggs by intraperitoneal injection of 5000 eggs. Fourteen days later, sensitized mice, four to six mice per group, were injected intravenously with 5000 eggs to induce synchronous pulmonary granulomas. Fourteen days after intravenous egg injection, mice were sacrificed and the draining mediastinal lymph nodes were removed. The lungs were processed for histology as described above.

Immunocytochemical Detection of ICAM-1 and VCAM-1

Lung tissue was removed, snap frozen, and cryosectioned. Antibodies for detection of ICAM-1 and VCAM-1 were purchased from PharMingen (San Diego, CA, USA). Immunocytochemistry to detect ICAM-1 and VCAM-1 expression was performed essentially following the manufacturer's instructions. Sections were incubated with Hamster IgG anti-mouse ICAM-1 (Clone 3E2), Hamster IgG standard (clone A19-3), Rat IgG2a anti-mouse VCAM-1 (Clone 429[MVCAM A]), and purified Rat IgG2a (clone R35-98). Bound antibodies were detected by incubation with biotinylated anti-hamster IgG (clones G70-204, G94-56) and anti-Rat IgG1/2a (clone G28-5). Sections were probed with streptavidin-peroxidase (StrepABC/Complex/HRP kit, Dako AS, Glostrup, Denmark) according to the manufacturer's instructions. 3,3′-Diaminobenzidine was used as substrate, and sections were counterstained with haematoxylin. Expression of ICAM-1 and VCAM-1 was evaluated double-blind using an arbitrary scoring system. 0, background staining comparable to irrelevant control antibody; +, weak sporadic staining; ++, unequivocal staining; and +++, unequivocal strong staining.

Immunization with Ovalbumin Protein Antigen

Mice were immunized intraperitoneally with 100 μg of ovalbumin/alum. At day 10, 20, and 30, these animals were administered intraperitoneally with 50 μg of ovalbumin/alum. After a further 10 days, mesenteric lymph node cells were prepared and cultured at 4×10^6 cells per ml in the presence of ovalbumin (25 μg/ml) for 5 days, or cultured at 2×10^6 cells per ml in the presence of anti-CD3 for up to 4 days. Anti-IL-4Rα antibody (Genzyme) at 0.5 μg/ml was added for the final 24 hr of culture. Supernatants were analyzed for cytokines using ELISA. Serum samples were assayed for ovalbumin antigen-specific immunoglobulin isotypes as detailed below.

Antigen-Specific Antibody ELISA

With the exception of antigen-specific IgE, ovalbumin-specific isotype responses were measured using ELISA by coating 96-well plates with ovalbumin at 5 μg/ml; bound immunoglobulin of diluted serum samples was detected using biotinylated monoclonal antiimmunoglobulin isotype detection antibodies (Becton Dickinson). Ovalbumin-specific serum IgE was determined using a two-step sandwich ELISA based on the method of van Halteren et al. (1997). ELISA plates were coated at 2 μg/ml with a monoclonal anti-mouse IgE antibody (Pharmingen clone R35-72). Serum samples were added and the plates incubated for 1 hr at room temperature. After washing, digoxigenin-coupled ovalbumin (DIG-OVA), prepared from a kit containing digoxigenin-3-*O*-methylcarbonyl-ε-amonocaproic acid *N*-hydroxy-succinimide ester (Boehringer Mannheim GmbH, Mannheim, Germany), was added and incubated for 1 hr at room temperature. After washing, anti-digoxigenin-Fab fragments coupled to peroxidase (Boehringer Mannheim GmbH, Mannheim, Germany) were used to detect bound antibody.

Helminth Infection

Individual mice were inoculated subcutaneously with 500 viable third-stage *N. brasiliensis* larvae. Animals were sacrificed 5, 10, and 15 days postinfection, the intestinal worm burdens determined, and serum taken. Jejunal tissue was removed and either snap frozen for mast cell protease assay or processed for histology as described above. In addition, mesenteric lymph node cells were harvested and stimulated in vitro at either 2.5×10^6 cells per ml with plate-bound anti-CD3 or at 5×10^6 cells per ml with 50 μg/ml of parasite antigens (NbES) (Lawrence et al., 1996) for 72 hr. Supernatants were harvested and analyzed for cytokines.

Total Serum Antibody ELISA

Serum immunoglobulins were assayed using sandwich ELISA. 96-well plates were coated with monoclonal antiimmunoglobulin isotype capture antibodies, and bound immunoglobulin of diluted serum samples was detected using biotinylated monoclonal antiimmunoglobulin isotype detection antibodies (Becton Dickinson). Concentrations were calculated using purified immunoglobulin isotypes as standards (Becton Dickinson).

Cytokine ELISA

Cytokine ELISA also utilized the sandwich format with capture and detection antibodies purchased from Becton Dickinson. ELISA were performed according to Becton Dickinson ELISA protocol.

Mouse Mast Cell Protease-1 ELISA

Mouse mast cell protease-1 levels in jejunal homogenates were assayed using a mMCP-1 ELISA kit purchased from Moredun Scientific. ELISA was performed according to the manufacturer's protocol.

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References

Bevilacqua, M.P. (1993). Endothelial-leukocyte adhesion molecules. Annu. Rev. Immunol. *11*, 767–804.

Bourette, R.P., Royet, J., Mouchiroud, G., Schmitt, E., and Blanchet, J.P. (1992). Murine interleukin 9 stimulates the proliferation of mouse erythroid progenitor cells and favors the erythroid differentiation of multipotent FDCP-mix cells. Exp. Hematol. *20*, 868–873.

Cohn, L., Homer, R.J., Marinov, A., Rankin, J., and Bottomly, K. (1997). Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. J. Exp. Med. *186*, 1737–1747.

Cohn, L., Homer, R.J., MacLeod, H., Mohrs, M., Brombacher, F., and Bottomly, K. (1999). Th2-induced airway mucus production is dependent on IL-4Rα, but not on eosinophils. J. Immunol. *162*, 6178–6183.

Costa, J.J., Weller, P.F., and Galli, S.J. (1997). The cells of the allergic response: mast cells, basophils, and eosinophils. JAMA *278*, 1815–1822.

Di Santo, J.P., Kuhn, R., and Muller, W. (1995). Common cytokine receptor gamma chain (γc)-dependent cytokines: understanding in vivo functions by gene targeting. Immunol. Rev. *148*, 19–34.

Emson, C.L., Bell, S.E., Jones, A., Wisden, W., and McKenzie, A.N.J. (1998). Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. J. Exp. Med. *188*, 399–404.

Fallon, P.G., and Dunne, D.W. (1999). Tolerisation of mice to *Schistosoma mansoni* egg antigens causes elevated type 1 and type 2 cytokine responses and increased mortality in acute infection. J. Immunol. *162*, 4122–4132.

Fallon, P.G., Smith, P., and Dunne, D.W. (1998). Type 1 and type 2 cytokine-producing mouse CD4+ and CD8+ T cells in acute *Schistosoma mansoni* infection. Eur. J. Immunol. *28*, 1408–1416.

Faulkner, H., Humphreys, N., Renauld, J.C., Van Snick, J., and Grencis, R. (1997). Interleukin-9 is involved in host protective immunity to intestinal nematode infection. Eur. J. Immunol. *27*, 2536–2540.

Faulkner, H., Renauld, J.C., Van Snick, J., and Grencis, R.K. (1998). Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris*. Infect. Immun. *66*, 3832–3840.

Finkelman, F., Shea-Donohue, T., Goldhill, J., Sullivan, C., Morris, S., Madden, K., Gause, W., and Urban, J. (1997). Cytokine regulation of host defence against parasitic gastrointestinal nematodes: lessons from studies with rodent models. Annu. Rev. Immunol. *15*, 505–533.

Foster, P., Hogan, S., Ramsay, A., Matthei, K., and Young, I. (1996). Interleukin-5 deficiency abolishes eosinophilia, airways hyperreactivity and lung damage in a mouse asthma model. J. Exp. Med. *183*, 195–201.

Frazer, K.A., Ueda, Y., Zhu, Y., Gifford, V.R., Garofalo, M.R., Mohandas, N., Martin, C.H., Palazzolo, M.J., Cheng, J.F., and Rubin, E.M. (1997). Computational and biological analysis of 680 kb of DNA sequence from the human 5q31 cytokine gene cluster. Genome Res. *7*, 495–512.

Friend, D.S., Ghildyal, N., Austen, K.F., Gurish, M.F., Matsumoto, R., and Stevens, R.L. (1996). Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. J. Cell Biol. *135*, 279–290.

Galli, S.J., and Hammel, I. (1994). Mast cell and basophil development. Curr. Opin. Hematol. *1*, 33–39.

Godfraind, C., Louahed, J., Faulkner, H., Vink, A., Warnier, G., Grencis, R., and Renauld, J.C. (1998). Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. J. Immunol. *160*, 3989–3996.

Grunig, G., Warnock, M., Wakil, A.E., Venkayya, R., Brombacher, F., Rennick, D.M., Sheppard, D., Mohrs, M., Donaldson, D.D., Locksley, R.M., et al. (1998). Requirement for IL-13 independently of IL-4 in experimental asthma. Science *282*, 2261–2263.

Lantz, C.S., Boesiger, J., Song, C.H., Mach, N., Kobayashi, T., Mulligan, R.C., Nawa, Y., Dranoff, G., and Galli, S.J. (1998). Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. Nature *392*, 90–93.

Lawrence, R., Gray, C., Osborne, J., and Maizels, R. (1996). *Nippostrongylus brasiliensis*: cytokine responses and nematode expulsion in normal and IL-4-deficient mice. Exp. Parasitol. *84*, 65–73.

Leder, L.D. (1979). The chloroacetate esterase reaction. A useful means of histological diagnosis of hematological disorders from paraffin sections of skin. Am. J. Dermatopathol. *1*, 39–42.

Longphre, M., Li, D., Gallup, M., Drori, E., Ordonez, C.L., Redman, T., Wenzel, S., Bice, D.E., Fahy, J.V., and Basbaum, C. (1999). Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. J. Clin. Invest. *104*, 1375–1382.

Louahed, J., Toda, M., Jen, J., Hamid, Q., Renauld, J.C., Levitt, R.C., and Nicolaides, N.C. (2000). Interleukin-9 upregulates mucus expression in the airways. Am. J. Respir. Cell Mol. Biol. *22*, 649–656.

Marsh, D.G., Neely, J.D., Breazeale, D.R., Ghosh, B., Freidhoff, L.R., Ehrlich-Kautzky, E., Schou, C., Krishnaswamy, G., and Beaty, T.H. (1994). Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science *264*, 1152–1156.

McKenzie, A., Li, X., Largaespada, D., Sato, A., Atsushi, K., Zurawski, S., Doyle, E., Milatovich, A., Francke, U., Copeland, N., et al. (1993). Structural comparison and chromosomal location of the human and mouse IL-13 genes. J. Immunol. *150*, 5436–5444.

McKenzie, G., Bancroft, A., Grencis, R., and McKenzie, A. (1998a). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Curr. Biol. *8*, 339–342.

McKenzie, G.J., Emson, C.L., Bell, S.E., Anderson, S., Fallon, P., Zurawski, G., Murray, R., and McKenzie, A.N.J. (1998b). Impaired development of Th2 cells in IL-13-deficient mice. Immunity *9*, 423–432.

McKenzie, G.J., Fallon, P.G., Emson, C.L., Grencis, R.K., and McKenzie, A.N.J. (1999). Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses. J. Exp. Med. *189*, 1565–1572.

Miller, H.R., Newlands, G.F., McKellar, A., Inglis, L., Coulson, P.S., and Wilson, R.A. (1994). Hepatic recruitment of mast cells occurs in rats but not mice infected with *Schistosoma mansoni*. Parasite Immunol. *16*, 145–155.

Mock, B.A., Krall, M., Kozak, C.A., Nesbitt, M.N., McBride, O.W.,

Renauld, J.C., and Van Snick, J. (1990). IL9 maps to mouse chromosome 13 and human chromosome 5. Immunogenetics *31*, 265–270.

Nawa, Y., Ishikawa, N., Tsuchiya, K., Horii, Y., Abe, T., Khan, A.I., Bing, S., Itoh, H., Ide, H., and Uchiyama, F. (1994). Selective effector mechanisms for the expulsion of intestinal helminths. Parasite Immunol. *16*, 333–338.

Nicolaides, N.C., Holroyd, K.J., Ewart, S.L., Eleff, S.M., Kiser, M.B., Dragwa, C.R., Sullivan, C.D., Grasso, L., Zhang, L.Y., Messler, C.J., et al. (1997). Interleukin 9: a candidate gene for asthma. Proc. Natl. Acad. Sci. USA *94*, 13175–13180.

Petit-Frere, C., Dugas, B., Braquet, P., and Mencia-Huerta, J.M. (1993). Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. Immunology *79*, 146–151.

Reiner, S., Zheng, S., Corry, D., and Locksley, R. (1993). Constructing polycompetitor cDNAs for quantitative PCR. J. Immunol. Methods *165*, 37–46.

Renauld, J.C., Goethals, A., Houssiau, F., Van Roost, E., and Van Snick, J. (1990). Cloning and expression of a cDNA for the human homolog of mouse T cell and mast cell growth factor P40. Cytokine *2*, 9–12.

Renauld, J.C., Druez, C., Kermouni, A., Houssiau, F., Uyttenhove, C., Van Roost, E., and Van Snick, J. (1992). Expression cloning of the murine and human interleukin 9 receptor cDNAs. Proc. Natl. Acad. Sci. USA *89*, 5690–5694.

Renauld, J.C., van der Lugt, N., Vink, A., van Roon, M., Godfraind, C., Warnier, G., Merz, H., Feller, A., Berns, A., and Van Snick, J. (1994). Thymic lymphomas in interleukin 9 transgenic mice. Oncogene *9*, 1327–1332.

Rogers, D.F. (1994). Airway goblet cells: responsive and adaptable front-line defenders. Eur. Respir. J. *7*, 1690–1706.

Sanderson, C.J. (1992). IL-5, eosinophils, and disease. Blood *79*, 3101–3109.

Shimbara, A., Christodoulopoulos, P., Soussi-Gounni, A., Olivenstein, R., Nakamura, Y., Levitt, R.C., Nicolaides, N.C., Holroyd, K.J., Tsicopoulos, A., Lafitte, J.J., et al. (2000). IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. J. Allergy Clin. Immunol. *105*, 108–115.

Temann, U.A., Geba, G.P., Rankin, J.A., and Flavell, R.A. (1998). Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. J. Exp. Med. *188*, 1307–1320.

Townsend, M.J., Fallon, P.G., Matthews, D.J., Jolin, H.E., and McKenzie, A.N. (2000). T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. J. Exp. Med. *191*, 1069–1076.

van Halteren, A.G.S., van der Cammen, M.J.F., Biewenga, J., Savelkoul, H.F.J., and Krall, G. (1997). IgE and mast cell responses on intestinal allergen exposure: a murine model to study the onset of food allergy. J. Allergy Clin. Immunol. *99*, 94–99.

Van Snick, J., Goethals, A., Renauld, J.C., Van Roost, E., Uyttenhove, C., Rubira, M.R., Moritz, R.L., and Simpson, R.J. (1989). Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). J. Exp. Med. *169*, 363–368.

Vink, A., Warnier, G., Brombacher, F., and Renauld, J.C. (1999). Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population. J. Exp. Med. *189*, 1413–1423.

Warren, K.S., and Domingo, E.O. (1970). Granuloma formation around *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum* eggs. Am. J. Trop. Med. Hyg. *19*, 291–304.

Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. Annu. Rev. Immunol. *17*, 255–281.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., and Donaldson, D.D. (1998). Interleukin-13: central mediator of allergic asthma. Science *282*, 2258–2261.

Wynn, T.A., Eltoum, I., Cheever, A.W., Lewis, F.A., Gause, W.C., and Sher, A. (1993). Analysis of cytokine mRNA expression during primary granuloma formation induced by eggs of *Schistosoma mansoni*. J. Immunol. *151*, 1430–1440.