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IL-25 Induces IL-4, IL-5, and IL-13 and Th2-Associated Pathologies In Vivo

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Summary

We have characterized a cytokine produced by Th2 cells, designated as IL-25. Infusion of mice with IL-25 induced IL-4, IL-5, and IL-13 gene expression. The induction of these cytokines resulted in Th2-like responses marked by increased serum IgE, IgG_1, and IgA levels, blood eosinophilia, and pathological changes in the lungs and digestive tract that included eosinophilic infiltrates, increased mucus production, and epithelial cell hyperplasia/hypertrophy. In addition, our studies show that IL-25 induces Th2-type cytokine production by accessory cells that are MHC class II^{high}, $CD11c^{dull}$, and $lineage^{-}$. These results suggest that IL-25, derived from Th2 T cells, is capable of amplifying allergic type inflammatory responses by its actions on other cell types.

Introduction

We have identified a cytokine (IL-25) that is structurally related to IL-17. (The interleukin-25 designation has been approved by the IUIS Subcommittee on Interleukin Nomenclature.) Other cytokines with structural homology to IL-17 have been identified as IL-17B and IL-17C (Li et al., 2000; Shi et al., 2000). Preliminary in vitro and in vivo data suggest that IL-17, IL-17B, and IL-17C have overlapping, albeit not identical, biological activities. For example, IL-17 induces the production of other cytokines and inflammatory mediators, including IL-1β, IL-6, IL-8, and TNFα, from a variety of cells (Fossiez et al., 1998; Jovanovic et al., 1998). IL-17B and IL-17C are able to induce the production of TNFα and IL-1β by a monocytic cell line (Li et al., 2000). Although gene expression studies indicate that the cellular sources of IL-17, IL-17B, and IL-17C may not be identical, their actions in vivo are similar (Li et al., 2000; Shi et al., 2000). Treatment of mice with IL-17 or IL-17B protein results in a similar phenotype, typified by neutrophil infiltration into tissue (Shi et al., 2000).

Although proinflammatory cytokines may be grouped into families based on structural homologies, subsequent investigations have shown that some cytokines within a given family may play unique roles in the generation of innate or acquired immune responses. IL-25 is structurally related to IL-17, and the human sequence has recently been described (Lee et al., 2000). Our studies show that the in vivo and in vitro biological activities of IL-25 are markedly different from those described for IL-17 and other IL-17-related family members. Treatment of mice with purified IL-25 resulted in the production of cytokines IL-4, IL-5, and IL-13, eosinophilia, increased serum Ig, and the development of striking histological changes in the lungs and the gastrointestinal (GI) tract.

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Results

Identification of IL-25

Using IL-17 and other family members for bait, we performed a BLAST search against the NCBI EST database and identified a unique mouse EST (IMAGE clone 419419) with significant sequence homology to IL-17. Full-length cloning of the mouse cDNA and human ortholog revealed ORFs encoding 169 and 161 residues for mouse and human, respectively. Analysis of these protein sequences identified the following overall conserved characteristics: an N-terminal leader sequence, a single potential N-linked glycosylation site, and calculated mature molecular weights of 17.5 (mouse) and 16.7 kDa (human). The mouse and human proteins have 10 conserved cysteine residues (11 total in the mouse) and are 80% identical (Figure 1).

Expression Analysis of IL-25

We examined various mouse cell lines and tissues for IL-25 gene expression, using a highly sensitive, quantitative PCR method. Analysis of a panel of cDNA libraries showed that only cDNA libraries made from T cells that had been stimulated for 7 days or for 3 weeks ("highly polarized") in the presence of IL-4/anti-IL-12 mAb had detectable IL-25 gene expression (Figure 2A). To confirm these results, naive $CD4^+$ T cells were isolated from BALB/c, B6, and 129 SvEv mice and polarized for 7 days in vitro in Th2-polarizing conditions (IL-4/anti-IL-12 mAb/anti-IFNγ mAb). The expression of IL-25 mRNA was detected in Th2-polarized T cells from all mouse strains, but not from freshly isolated naive $CD4^+$ T cells (data not shown), which confirmed the results from the cDNA library panel. Despite its association with Th2 cells, IL-25 mRNA expression was not detectable in the spleens of wild-type (WT) mice (Figure 2B). Low levels of IL-25 mRNA expression were found in various tissues (Figure 2B), with the highest expression in the GI tract and the uterus. As T cells can be found throughout the entire GI tract and other mucosal tissues, activated Th2 cells within those tissues may be the source of IL-25 mRNA. However, it remains possible that other cell types may express the IL-25 gene as well.

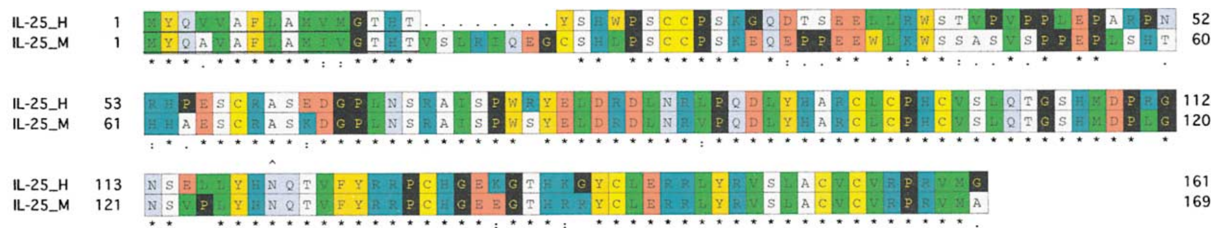

Figure 1. Alignment of Amino Acid Sequences of Mouse and Human IL-25

Human and mouse IL-25 protein sequences were aligned using ClustalX. The predicted leader cleavage site is marked with an arrow. The predicted N-linked glycosylation site is marked with "^". Conserved and identical residues are indicated by ":" and "*", respectively. An amino acid coloring scheme correlates chemically similar residues as follows: green (hydrophobic), red (acidic), blue (basic), yellow (C), orange (aromatic), black (structure breaking), purple (amido), and gray (small).

IL-25 Protein Treatment of Mice Induces Splenomegaly, Eosinophilia, and Serum IgE

To gain a better understanding of the biological role of IL-25 in vivo, we injected WT mice intraperitoneally (i.p.) with purified IL-25 protein or saline and monitored the mice for changes in hematopoietic parameters. Mice treated daily with 10 μg of IL-25 for 10 days developed splenomegaly with significantly higher numbers of eosinophils but not neutrophils (Figures 3A and 3B). The number of splenic plasma cells was also increased (Figure 3B). In addition, spleens from IL-25-treated mice had a 20-fold higher number of myeloid colony forming cells (CFU-c) as compared to control spleens, indicating a mobilization of hematopoietic progenitor cells to the spleens of IL-25-treated animals (data not shown). The increased numbers of splenic eosinophils were mirrored by an increase in eosinophils in the blood. To determine the dose and time course of the eosinophilia seen in response to IL-25 treatment, mice were given 0.1–10 μg IL-25 daily, and blood samples were taken at various time points. As seen in Figure 3C, as little as 0.1 μg IL-25 caused eosinophilia in the peripheral blood by day 4 of treatment. The blood eosinophilia reached a peak at day 7 at all doses of IL-25 given. The absolute number of peripheral blood neutrophils was unaffected by IL-25 at any dose or time point (Figure 3C). These data show that IL-25 can induce the mobilization of eosinophils into the blood. Importantly, these hematopoietic changes in response to IL-25 treatment were seen in three different strains of WT mice (C57BL/6, BALB/c, and 129 SvEv), and these data indicate that observed immunologic changes were not due to a strain-specific effect of IL-25 (data not shown).

Th2-type inflammatory responses are characterized by recruitment of eosinophils as well as increased serum IgE and IgG_1 (Romagnani, 1992). Therefore, we evalu-

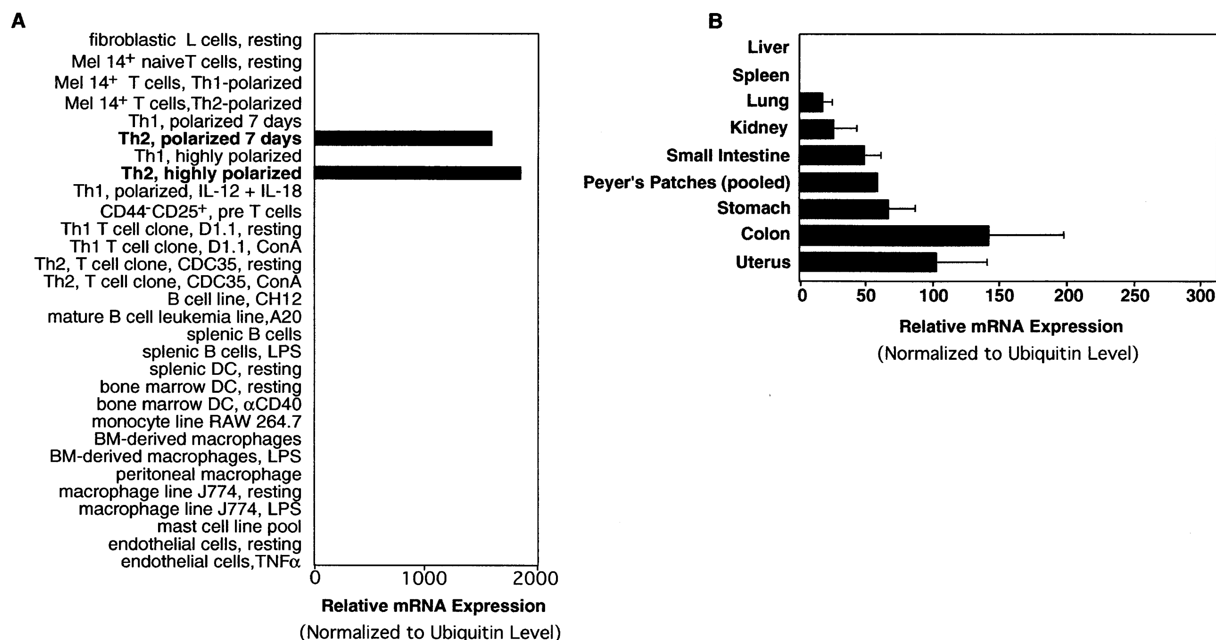

Figure 2. Gene Expression of IL-25 in Various Cell Lines and Tissues

(A) The relative mRNA expression of IL-25 in 20 ng of cDNA from various cell line libraries.

(B) The relative mRNA expression of IL-25 in 50 ng of cDNA from tissues from BALB/c mice. Tissues were taken from four mice per group, and the amount of IL-25 mRNA was quantified for each individual tissue, except for Peyer's Patches, which were pooled due to the small amount of tissue.

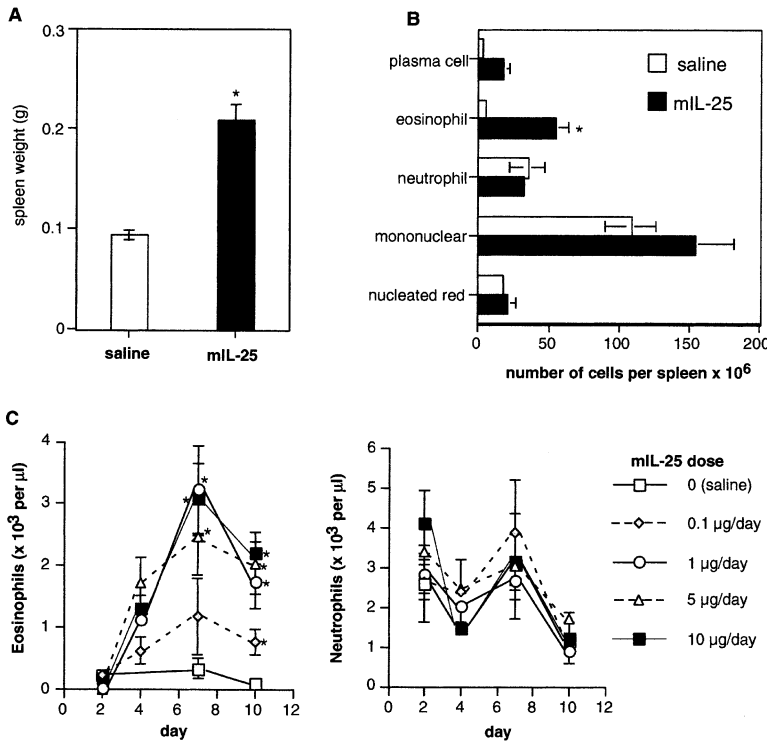

Figure 3. In Vivo Treatment with IL-25 Protein Results in Splenomegaly and Eosinophilia

(A and B) C57BL/6 mice ($n = 4$ per group) were injected i.p. daily, starting on day 1, with saline or 10 μg IL-25 for 10 days. Spleens were harvested, weighed, and then made into single-cell suspensions to determine total cellularity. Relative populations were determined microscopically with Wright's-Giemsa-stained cytospins.

(C) C57BL/6 mice were treated for 10 days with varying doses of IL-25 or with saline ($n = 5$ per group). At days 2, 4, 7, and 10, blood samples were taken for total white blood cell count, and the relative populations were enumerated from blood smears stained with Wright's-Giemsa. (*, $p < 0.05$)

ated circulating Ig levels in mice receiving IL-25 (Figure 4). Mice treated daily with 5 μg IL-25 had significantly higher serum IgE and IgG_1. Interestingly, circulating IgA was greatly increased in IL-25-treated animals (Figure 4). In addition, the amount of IgA in the intestinal contents was higher with IL-25 treatment (2.66 ± 0.46 μg/ml versus 6.89 ± 0.75 μg/ml in saline-treated versus IL-25-treated mice, respectively; $p = 0.0014$).

IL-25 Induces Gene Expression of Th2-Associated Cytokines

Increased circulating and infiltrating eosinophils are associated with the production of IL-5, while IgE, IgG_1, and IgA synthesis are associated with IL-4 and IL-13 (Bost et al., 1996; Chomarat and Banchereau, 1998; Roboz and Rafii, 1999). Therefore, we tested whether IL-25 treatment might induce the expression of Th2-associated cytokines in vivo. Total RNA was isolated from various tissues from mice treated for 10 days with either saline or 10 μg IL-25 and then tested for the presence of IL-4, IL-5, and IL-13 mRNA by quantitative PCR analysis. IL-25 treatment strongly induced the gene expression of IL-13 in all tissues tested, including the spleen, stomach, small intestine, kidney, liver, lung, and colon (Figure 5A and data not shown). The mRNA expression of both IL-4 and IL-5 was significantly higher in the spleens of IL-25-treated mice, but varied in other tissues. We also examined the gene expression of other cytokines and chemokines. The spleen, stomach, and small intestine of IL-25-treated mice had significantly increased expression of IL-6 and eotaxin when compared to tissues from saline-treated mice (data not shown). There was no change in the gene expression of IL-1α, TNFα, IL-10, or IFNγ with IL-25 treatment (data not shown). Moreover, there was no increase in IL-25 mRNA expression in mice treated with IL-25, suggesting IL-25 does not induce its own gene expression (data not shown). Together these data indicate that IL-25 treatment preferentially induces the gene expression of Th2-associated cytokines.

To confirm that IL-25 could induce the production of Th2 cytokines, we tested the ability of IL-25 to stimulate the production of IL-4, IL-5, and/or IL-13 in vitro. When $CD4^+CD45RB^{high}$ ("naive") or $CD4^+CD45RB^{low}$ ("memory") T cells were stimulated with anti-CD3/anti-CD28 mAbs in the presence of IL-25, the production of IL-4, IL-5, and IL-13 by these cells was unaffected (data not

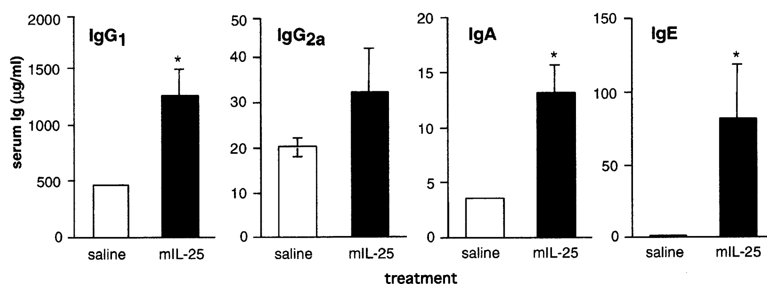

Figure 4. IL-25 Induces an Increase in Serum IgG_1, IgA, and IgE

C57BL/6 mice ($n = 4$ per group) were injected daily i.p. with either saline or 5 μg/day of IL-25 protein for 10 days. Blood was collected on day 10 to determine serum isotype levels by sandwich ELISA. Results shown are representative of two independent experiments. (*, $p < 0.05$)

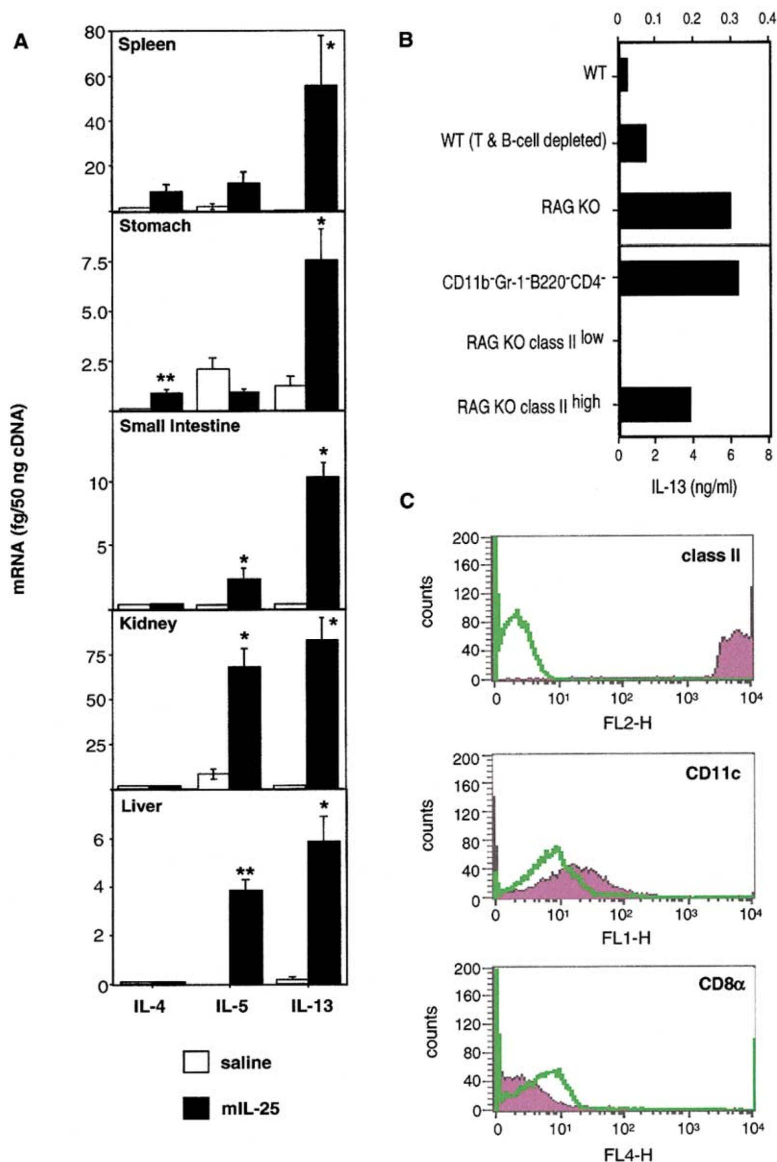

Figure 5. IL-25 Induces IL-4, IL-5, and IL-13 Gene Expression and Protein Production from a Nonlymphoid Accessory Cell

(A) C57BL/6 mice (n = 3–5 per group) were treated daily with 10 μg IL-25 or saline for 10 days and then organs were harvested. Total RNA was isolated from individual tissues, and the amount of IL-4, IL-5, and IL-13 mRNA was quantified by PCR as described in Experimental Procedures. The results shown are typical of three independent experiments. (*, $p < 0.05$; **, $p < 0.01$)

(B) Unfractionated splenocytes from C57BL/6 mice (WT), T and B cell-depleted splenocytes from WT mice (T & B cell depleted) and RAG KO mice were cultured in the presence or absence of optimal concentrations of IL-25 (10–50 ng/ml) as described in Experimental Procedures. Splenocytes from RAG KO mice treated with IL-25 were depleted of CD11b$^+$, Gr-1$^+$, B220$^+$, and CD4$^+$ cells (CD11b$^-$Gr-1$^-$B220$^-$CD4$^-$) or sorted for high or low expression of MHC class II (RAG KO class II^{low}; RAG KO class II^{high}) and were cultured in the presence of optimal concentrations of IL-25. Cell-free supernatants were assayed for IL-13 by sandwich ELISA. The results shown are typical of two to four independent experiments.

(C) RAG KO mice were treated with 5 μg/mouse IL-25 for 4–5 days, and MHC class II^{high} expressing cells were sorted from their spleens (>95% purity). Sorted cells were then analyzed by FACS for expression of CD11c and CD8α. Green line denotes isotype control staining.

shown). Furthermore, IL-25 did not induce IL-4 production by purified CD19$^+$CD11b$^+$ or CD19$^+$CD11b$^-$ B cells stimulated in the presence of LPS and/or anti-CD40 mAb. When unfractionated splenocytes from WT mice were cultured in the presence of 10 ng/ml IL-25, barely detectable amounts of IL-13 were produced (Figure 5B, upper panel). Furthermore, the cells producing IL-13 in response to IL-25 were non-T/non-B cells, as more IL-13 was found in supernatants from cultures containing either WT splenocytes depleted of T and B cells or splenocytes from immunodeficient (RAG KO) mice (Figure 5B, upper panel). As little as 0.1 ng/ml IL-25 induced the production of IL-13 protein from RAG KO splenocytes, with optimal stimulation at 10 ng/ml (data not shown). As similar results were obtained for the production of IL-5 (data not shown), our results indicate that IL-25 is able to induce the production of at least two Th2-associated cytokines from a non-T/non-B cell population. Surprisingly, no IL-4 was detected under these conditions.

To further characterize which non-T/non-B cells may be producing IL-5 and/or IL-13 in response to IL-25 in vitro, we examined various cell types present in the spleens of RAG KO mice. Purified monocytes (CD11b$^+$ splenocytes and macrophage cell lines), splenocytes from IL-5 transgenic mice (enriched for eosinophils), and purified NK1.1$^+$CD3$^-$ cells were unresponsive to IL-25 (data not shown). As the IL-25-responding cells were obviously extremely rare, even in a RAG KO spleen, we attempted to enrich for these cells by treating RAG KO mice with IL-25 in vivo (5 μg/day for 4–5 days). The depletion of CD11b$^+$, Gr-1$^+$, B220$^+$, and CD4$^+$ cells from the spleens of IL-25-treated RAG KO mice resulted in an enrichment of cells that produced IL-13 (Figure 5B, lower panel) and IL-5 (data not shown). Spleen cells from IL-25-treated mice were then divided into two populations by flow cytometry based on their expression of MHC class II molecules: class II^{low} and class II^{high}. As seen in Figure 5B, class II^{high} cells, but not class II^{low} cells, produced significant amounts of IL-13 upon exposure

to IL-25. FACS analysis of the IL-25 responders showed these cells to be MHC class II^{high}, $CD11c^{dull}$, $F4/80^{low}$, and $CD4^{-}CD8\alpha^{-}$ (Figure 5C and data not shown).

IL-25 Treatment Induces Pathological Changes in the Lung and the Digestive Tract

In addition to hematological changes, we also observed gross changes in IL-25-treated mice. These changes included enlarged stomachs and duodenums that were full of mucus and bile. Therefore, we microscopically examined multiple tissues from saline and IL-25-treated animals. In mice treated for 10 days with 5–10 μg IL-25, striking histological changes were found in mucosal tissues, including the lungs, esophagus, stomach, small intestine, and large intestine (Figure 6 and data not shown).

In the lung, the vascular changes were observed in the medium and small muscular arteries (Figures 6A–6C). Changes included minimal to moderate medial hypertrophy; the presence of eosinophils within the vascular lumen; and infiltrates of eosinophils and/or mononuclear cells beneath the endothelium, within the vessel wall, and adjacent to the vessel. In contrast to the vascular changes, which involved more distal vessels, the changes in the airways were primarily restricted to bronchi and larger bronchioles (Figures 6D and 6E). The epithelium lining the airways was hypertrophied and appeared to contain large amounts of mucus. Periodic acid-Schiff (PAS) and alcian blue stains highlighted the presence of large amounts of mucus within the lining epithelium (goblet cells are not usually seen in the healthy mouse lung). In some mice, mucus filled the airway lumina. Epithelial cells also sometimes contained eosinophilic cytoplasmic inclusions. Eosinophilic, occasionally crystalline, material was also observed within airway lumina and within alveolar macrophages.

In the digestive tract, epithelial hyperplasia was evident in the esophagus (Figures 6F and 6G) and nonglandular stomach. Epithelial cells often contained prominent eosinophilic cytoplasmic inclusions, similar to those seen in the respiratory tract epithelium. In the glandular stomach, vacuolation of epithelial cells in the gastric glands was observed (Figures 6H and 6I), and eosinophilic cytoplasmic inclusions were seen in pyloric epithelial cells. The eosinophilic material in the lungs and digestive tract did not stain with PAS or alcian blue. However, the vacuolated cells in the glandular stomach were PAS- and alcian blue-positive, suggesting that they were mucous cells. Inflammatory infiltrates of eosinophils, neutrophils, and mononuclear cells were seen in the epithelium and lamina propria of the esophagus and stomach and sometimes in the submucosa and serosa. In the small and large intestines, the goblet cells were hypertrophied and hyperplastic (Figures 6J and 6K), and the lumen of the intestines sometimes contained increased mucus. Brunner's glands in the duodenum were enlarged and vacuolated. Furthermore, the epithelia of the large biliary duct in the liver and the large pancreatic ducts were often vacuolated and contained eosinophilic cytoplasmic inclusions.

The incidence and severity of the changes were dose related, and as little as 0.1 μg IL-25 for 10 days could induce some histological changes in the lungs and small intestine (data not shown). No histological changes were observed in nonmucosal tissues, including the heart, thyroid, skeletal muscle, and kidney. Thus, though IL-25 treatment can induce cytokine gene expression in many different tissues in vivo, only mucosal tissues responded with pathological changes.

IL-25 Mediates Its Effects In Vivo through the Induction of IL-4, IL-5, and IL-13

The ability of IL-25 to increase mucus production, serum IgE, IgG_1, and IgA production, and the number of circulating eosinophils could be explained by its ability to induce the expression of IL-4, IL-5, and IL-13. To test this hypothesis, we looked at the ability of IL-25 to affect hematopoietic and histological changes in various types of mutant mice. To be able to look at sufficient numbers of mice over an extended period of time, we infected mice with an adenoviral construct containing the IL-25 gene (Ad-IL-25). Control mice were infected with the same adenoviral construct lacking the IL-25 gene (Ad-control). As summarized in Table 1, infection of WT mice with Ad-IL-25 resulted in the same changes observed in WT mice treated with IL-25 protein: eosinophilia, increased serum Ig, increased gene expression of Th2-associated cytokines, and pathological changes in mucosal tissues. Although the histological changes in mice receiving Ad-IL-25 and IL-25 protein were similar, the findings tended to be more florid in mice receiving Ad-IL-25, and these changes lasted for up to 30 days postinfection (Figures 6L and 6M). In addition, perivascular fibrosis was seen in the lungs of mice receiving Ad-IL-25, but not mice receiving IL-25 protein (data not shown). Ad-IL-25-infected immunodeficient (RAG KO) mice showed the same responses as WT mice, except that no IL-4 mRNA was detectable in these mice (Table 1). These data showed that the cells producing IL-5 and IL-13 in response to IL-25 were neither T nor B cells and are in agreement with our in vitro studies using lymphoid-deficient splenocytes (Figure 5B).

To determine the roles of IL-4 and IL-13 in the effects of IL-25 treatment, IL-4Rα KO mice were infected with Ad-IL-25. The IL-4Rα chain is part of both the IL-4 and IL-13 receptor complexes (Hilton et al., 1996; Zurawski et al., 1995). Interestingly, although the IL-4Rα KO mice showed increased mRNA expression of IL-4, IL-5, and IL-13 and developed peripheral blood eosinophilia, they did not develop any histological changes in the lung or digestive tract (Table 1). Therefore, signaling through the IL-4Rα chain is necessary for the development of the increased mucus production and epithelial changes, but not for the development of eosinophilia, in response to IL-25. To further differentiate the relative contributions of IL-4 and IL-13 as mediators of the effects of IL-25, we infected IL-4 KO and IL-13 KO mice with the adenoviral constructs. Ad-IL-25-infected IL-4 KO mice had no increase in serum IgA, IgE, or IgG_1, showing a dependence on IL-4 gene expression for the production of these Ig isotypes in response to IL-25 (Table 1). Interestingly, Ad-IL-25-infected IL-4 KO mice did not have any pulmonary vascular changes, but did have epithelial changes in the lungs and GI tract that were equivalent to those seen in WT mice. These results suggest that IL-4 may have contributed to the pulmonary vascular changes, but not

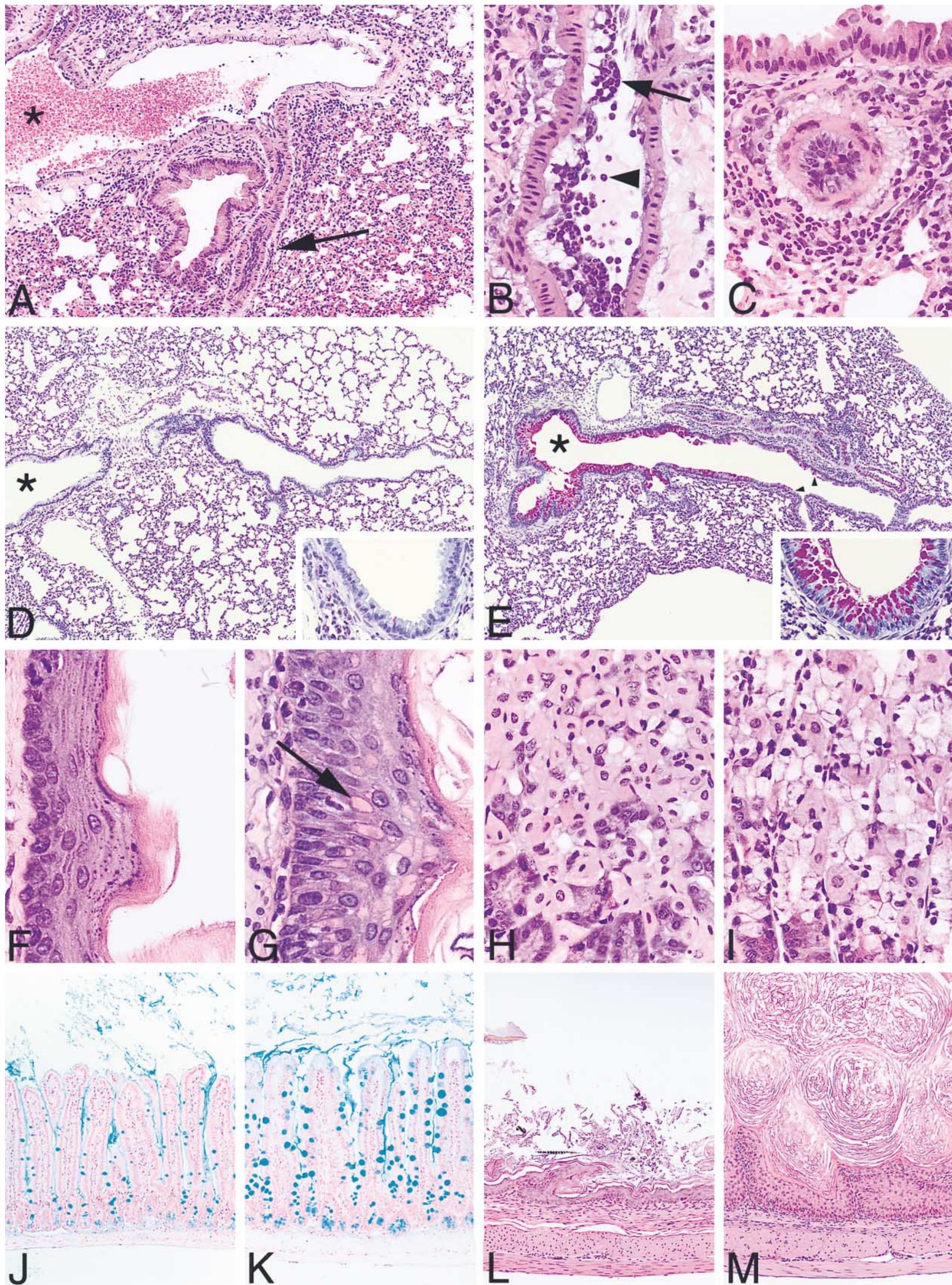

Figure 6. Microscopic Changes in Mice Receiving 10 μg IL-25 Protein for 10 Days or Mice Infected with Ad-IL-25, 30 Days Postinfection

(A–C) Pulmonary changes in mice receiving IL-25 protein (hematoxylin and eosin [H&E]). (A) Vascular changes are localized to medium and small muscular arteries. The larger muscular artery (*) has no changes, while the smaller branch (arrow) has mild medial hypertrophy and infiltrates of inflammatory cells (×80). (B) Higher magnification of vascular changes in muscular arteries. Numerous eosinophils within the

Table 1. IL-13 Mediates the Histological Changes Seen in Mice Infected with Ad-IL-25 Viral Constructs or Infused with IL-25 Protein

Mice	Blood Eosinophils[a]	Serum Ig[a]			Gene Expression[a]			Histological Changes
		IgA	IgE	IgG_1	IL-4	IL-5	IL-13	
WT	11x**	3x**	348x**	8x**	11x*	14x*	525x*	present
RAG 1 KO	6x**	NA	NA	NA	NC	5x**	172x**	present
IL-4Rα KO	11x**	NC	NC	NC	12x*	31x**	753x**	absent
IL-4 KO	10x**	NC	NC	NC	NA	12x*	96x*	present
IL-13 KO	3x*	NC	7x**	5x**	2x*	5x*	NA	absent
WT + αIL-5 mAb	NC	3x*	1337x*	17x**	9x**	3x*	14x**	present

WT (BALB/c, C57BL/6, and 129 SvEv) mice, C57BL/6 RAG 1 KO mice, BALB/c IL-4Rα KO, BALB/c IL-4KO mice, and 129 SvEv IL-13KO mice received i.v. 5×10^{10} virus particles of either Ad-control or Ad-mIL-25. WT C57BL/6 mice were infused daily with IL-25 protein or saline. IL-25-treated mice wre also infused weekly with 2 mg of anti-IL-5 mAb or isotype control.
NA, not applicable; NC, no significant change, *$p < 0.05$; **$p < 0.01$.
[a] Values represent fold increases above Ad-control-treated mice or, for the anti-IL-5 mAb experiment, saline-treated control mice.

to the epithelial changes, observed in mice treated with IL-25. IL-13 KO mice that were infected with Ad-IL-25 showed increased serum IgE and IgG_1, but not IgA, suggesting that increased IgA production was dependent on IL-13 (Table 1). Furthermore, Ad-IL-25-infected IL-13 KO mice did not develop any changes in the lungs or digestive tract, indicating that the induction of IL-13 by IL-25 was critical for the histological changes seen in the lungs and the digestive tract.

As blood eosinophilia was evident in all mutant mouse strains infected with Ad-IL-25 (Table 1), we tested the dependence of eosinophilia on the induction of IL-5 by IL-25. Mice were cotreated with IL-25 protein and either isotype control mAb or anti-IL-5 mAb. Anti-IL-5 mAb completely abolished the presence of circulating eosinophils in the blood in WT mice that were cotreated with 5 μg/day IL-25 for 10 days ($1.7 \pm 0.8 \times 10^3$ versus $0.0 \pm 0.0. \times 10^3$ eosinophils per μl blood in IL-25 protein + isotype control mAb-treated versus IL-25 + anti-IL-5 mAb-treated mice, respectively; see Table 1). The anti-IL-5 mAb/IL-25 cotreated mice developed histological changes that were equivalent to isotype-control-treated animals, with the exception that no eosinophilic infiltrate was seen in the analyzed tissues. Anti-IL-5 mAb treatment also did not affect the development of increased serum Ig and cytokine gene expression in response to IL-25 (Table 1). In summary, the effects of IL-25 treatment in vivo are indirectly mediated through the induction of the Th2-associated cytokines IL-4, IL-5, and IL-13.

Discussion

Although IL-25 is structurally related to IL-17, its biological effects differ dramatically from previously described members of the IL-17 family. The IL-17 gene is expressed in activated $CD4^+$ T cells and is undetectable in whole tissues (Fossiez et al., 1998). IL-17 induces splenomegaly due to mobilization of bone marrow progenitor cells to the spleen and the expansion of neutrophils (Schwarzenberger et al., 1998). Furthermore, intratracheal injections of IL-17 and intraperitoneal injections of IL-17B induce neutrophil recruitment to the lung and the peritoneum, respectively (Linden et al., 2000; Shi et al., 2000). IL-17, as well as perhaps IL-17B and IL-17C, mediates its effects on granulopoiesis and inflammation by the induction of proinflammatory cytokines, such as IL-1β, IL-6, GROα, and TNFα (Linden et al., 2000; Schwarzenberger et al., 2000; Witowski et al., 2000). IL-25, on the other hand, is expressed in activated Th2 T cells and induces a very different type of inflammatory response in vivo.

Treatment of mice with IL-25 resulted in the induction of a different set of cytokine genes compared to IL-17. Although IL-25 induced a small amount of IL-6 gene expression, mRNA levels of IL-1β, TNFα, G-CSF, and GM-CSF were unchanged. The most significant gene expression induced in response to IL-25 was IL-4, IL-5, and IL-13. Furthermore, treatment of mice with IL-25 resulted in blood eosinophilia and splenomegaly due to the mobilization of hematopoietic progenitors and the expansion of eosinophils. Increased serum IgE, IgG_1, and IgA isotypes were found in IL-25-treated mice. Finally, exposure to IL-25 caused striking histological changes in the lungs and GI tract, including eosinophilic and mononuclear infiltrates, increased mucus production, and epithelial cell hyperplasia and hypertrophy.

Our results with cytokine-deficient mice clearly show that the induction of IL-4, IL-5, and IL-13 protein by IL-25

lumen (arrowhead) and beneath the endothelium (arrow) (×230). (C) Mononuclear cells and a few eosinophils almost occlude the arterial lumen.

(D and E) Epithelial changes in the lungs. Proximal airway (*) (periodic acid-Schiff [PAS] stain, ×40 [inset ×120]). (D) Lung from mouse that received saline. Inset: higher magnification of bronchiole. (E) Lung from mouse that received IL-25 protein. Intense PAS-positive staining in the proximal airway epithelium, extending distally to the small arrowheads. Inset: higher magnification of bronchiole.

(F and G) Esophageal changes (H&E, ×375). (F) Esophagus from mouse receiving saline. (G) Esophagus from mouse receiving IL-25 protein. Epithelial hyperplasia with eosinophilic cytoplasmic inclusions (arrow).

(H and I) Stomach changes (H&E, ×60). (H) Glandular stomach from mouse receiving saline. (I) Glandular stomach from mouse receiving IL-25 protein. Vacuolation of mucous cells in the gastric glands.

(J and K) Jejunal changes (alcian blue, ×75). (H) Jejunum from mouse receiving saline. (I) Jejunum from mouse receiving IL-25 protein. Hypertrophy and hyperplasia of goblet cells.

(L and M) Changes in nonglandular stomach (H&E, ×60). (L) Nonglandular stomach from mouse receiving Ad-control virus. (M) Nonglandular stomach from mouse receiving Ad-IL-25. Epithelial hyperplasia and marked hyperkeratosis.

is responsible for the observed immunologic changes. The increased serum IgE and IgG_1 seen in IL-25-treated mice was directly due to an increased production of IL-4, as Ad-IL-25-infected IL-4-deficient animals did not have detectable changes in serum IgE or IgG_1. In mice, IL-4 is both necessary and sufficient to promote Ig isotyping switching to IgE and IgG_1 (Chomarat and Banchereau, 1998; Corry, 1999; Pène et al., 1988). Interestingly, elevated serum IgA concentrations were seen in WT mice infected with Ad-IL-25, but not in IL-4 KO or IL-13 KO mice. These data suggest that both IL-4 and IL-13 are necessary for IgA production, a result consistent with studies by others (Bost et al., 1996; DeKruyff et al., 1993; Yamamoto et al., 1996). The observed blood eosinophilia in IL-25-treated mice was directly related to the induction of IL-5, as anti-IL-5 mAb cotreatment effectively suppressed the numbers of circulating eosinophils as well as eosinophilic infiltrates in the GI tract and the lungs. IL-5 is known to be critical for the differentiation and the release of eosinophils from the bone marrow (Karlen et al., 1998; Roboz and Rafii, 1999). IL-25-treated mice also had increased eotaxin gene expression. Eotaxin is a key chemokine for the recruitment of eosinophils to tissues (Rothenberg, 1999) and may be responsible for the increased presence of eosinophils in the lungs and GI tract of IL-25-treated mice. As eotaxin gene expression can be induced by IL-4 and IL-13, IL-25 may not induce eotaxin production directly (Li et al., 1999).

The induction of IL-13 protein expression is the primary mediator for the striking histological changes seen in the lungs and digestive tracts in IL-25-treated animals. Only IL-25-treated mice that were deficient in IL-13 or a component of the IL-13 receptor (IL-4Rα chain) did not develop any microscopic changes. IL-13 has been shown to be necessary for the increased mucus production and airway hyperresponsiveness that characterize experimental asthma models (Corry, 1999, Grünig et al., 1998; Wills-Karp et al., 1998). Furthermore, mice with lung-specific transgene expression of IL-13 have a histological phenotype similar to the lungs from IL-25-treated mice (Zhu et al., 1999). The lungs of pulmonary-IL-13-transgenic mice have mononuclear and eosinophilic inflammatory infiltrates, epithelial cell hypertrophy, greatly increased mucus production, and eosinophilic crystals. Similar eosinophilic crystals have been previously reported and may be composed of a T cell-derived eosinophilic chemotactic factor, Ym1 (Ennulat et al., 1998; Guo et al., 2000). While the local production of IL-13 resulted in significant histological changes in the lungs of transgenic mice, IL-25 treatment induced the systemic production of IL-13, which resulted in pathological changes in the GI tract as well as in the lungs.

Interestingly, RAG KO mice treated with IL-25 had the same hematological and histological changes as did WT mice. Because IL-25-treated RAG KO mice had increased gene expression of IL-5 and IL-13, it appeared that there were non-T/non-B cells that responded to IL-25 by producing Th2-associated cytokines. Our studies with RAG KO splenocytes show that the IL-25 responder is an accessory cell that is MHC class $II^{high}CD11c^{dull}F4/80^{low}CD8\alpha^-CD4^-$. While the role of this accessory cell in vivo is currently unknown, the high expression of MHC class II suggests that this cell may be able to present antigen to T cells. As many different tissues respond to IL-25 treatment by increased IL-5 and IL-13 gene expression, it is likely that the identified accessory cell is widely distributed among different tissues. Further studies of this cell type, its tissue distribution, and its response to IL-25 are currently being pursued. It remains possible that other cells types may respond to IL-25 by producing Th2 cytokines. Other innate immune cells that are known to be able to produce IL-5 and/or IL-13 include NK cells, mast cells, eosinophils, and basophils (Chomarat and Banchereau, 1998; Hoshino et al., 1999; Roboz and Rafii, 1999). Our studies with purified NK cells, monocytes, and splenocytes enriched for eosinophils indicate that they do not produce either IL-5 or IL-13 in response to IL-25 exposure in vitro. Interestingly, spleens from WT, but not RAG KO, mice had significantly increased IL-4 gene expression, suggesting that T cells and/or B cells were expressing IL-4 mRNA in response to IL-25. However, under the in vitro conditions we used, IL-25 failed to induce IL-4 production by unfractionated WT splenocytes, purified memory or naive $\alpha\beta TCR^+CD4^+$ T cells, or purified $CD19^+CD11b^-$ or $CD19^+CD11b^+$ B cells. Other potential sources of IL-4, including $\gamma\delta TCR^+$ T cells and NK-T cells, are currently under investigation.

Our studies indicate that IL-25 may be a key cytokine for the development or the augmentation of Th2-associated diseases. IL-25 induces MHC class II-expressing accessory cells to produce IL-5 and IL-13. These IL-25-responsive cells may ultimately aid in the further development of the $CD4^+$ Th2 cells that are key for the development of asthma and other allergic reactions, as well as antiparasitic responses (Corry, 1999; Romagnani, 1992). Furthermore, as IL-25-responsive cells are distributed among many different organs, IL-25 may be an important mediator of the development of systemic Th2 responses. In humans, for example, there is an association between the development of asthma and other types of allergies with the influx of eosinophils into the esophagus (Furuta, 1998; Harding, 1999; Walsh et al., 1999). Furthermore, a mouse model of experimental esophagitis shows an influx of eosinophils into the esophagus and the lungs after exposure to a respiratory allergen (Mishra et al., 2001). It is possible that, by producing IL-25, localized $CD4^+$ Th2 cells could induce systemic Th2 inflammatory responses. Further study of IL-25 will bring new perspectives on the development of antiparasitic and allergic responses.

Experimental Procedures

Cloning of Mouse and Human IL-25

Nested PCR was performed with gene-specific primers and vector primers using a mouse Th2 cDNA library. The sequence of this PCR fragment yielded the 5′ end missing in the original clone, and a subsequent PCR using 5′ primer ATGTACCAGGCTGTTGCATTC TTG and 3′ primer CTAAGCCATGACCCGGGGCCGCACACACAC ACA generated the full-length fragment. This method was also used to PCR the full-length human fragment. Full-length cDNAs were cloned into the TOPO vector (Invitrogen), and the sequence was verified using an ABI 377 or 373 genetic analyzer.

Recombinant Adenovirus and Protein Production

Using PCR mutagenesis on the IL-25 TOPO template, a BglII site was introduced using a 5′ vector primer CATGATTACGCCAAGCTT

GGTACCGAGATCTGATCCACTAGTAACGGCCGCCAGTG and 3′ T7 vector primer. This PCR product was digested with BglII and EcoRV and cloned into the BglII- and PmeI-digested adenovirus transfer vector. The vector and recombinant adenovirus production were as described (Hoek et al., 2000). 5×10^8 293 cells (Quantum Biotechnologies Inc., Montreal, Canada) were infected with MOI ~10 Ad-IL-25 in 1 l CMF-1 medium (CellWorks, San Diego, CA) and incubated for 5 days in a cell factory (Nalgen Nunc Int., Naperville, IL). Culture medium was dialyzed (membrane tubing, MW: 6,000–8,000, Spectrum Laboratories, Inc., Rancho Dominguez, CA) versus 50 mM Tris-HCl (pH 8.0), 1 mM EDTA (Buffer A) and passed over HiTrap Q (Pharmacia, Uppsala, Sweden) to remove virus and many contaminating proteins. IL-25 in the flow-through was purified by Heparin Sepharose affinity chromatography (Pharmacia) in Buffer A with NaCl gradient elution. Peak fractions of >95% pure IL-25 were pooled and dialyzed into phosphate-buffered saline and quantified by PAGE and Coomassie Blue staining using lysozyme as a standard. LPS (endotoxin) levels (<1 eu/ml) were determined (BioWhittaker Limulus Amebocyte Lysate QCL-1000 pyrogen testing kit, Walkersville, MD).

RNA Isolation and Quantification of mRNA Expression

For the determination of IL-25 gene expression in cell lines, previously described cDNA libraries were used (Halfon et al., 1998). For quantification of IL-25 and other cytokines in whole mouse tissues, total RNA was isolated from whole tissues using Qiagen Rneasy columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (5 μg) was treated with DNase I to remove any contaminating genomic DNA (Ambion, Austin, TX) and then reverse transcribed into cDNA using random hexamers (Promega, Madison, WI). Then gene expression was determined by a method for real-time quantitative PCR using the ABI 7700 sequence detector system (Perkin Elmer Applied Biosystems, Foster City, CA). Briefly, 50 ng total cDNA was in a reaction volume of 25 μl that contained final concentrations of 1× PCR buffer, 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 4 mM $MgCl_2$, 1.25 U AmpliTaq DNA polymerase, 0.5 U Amp-Erase uracil-N-glycocylase, 900 nM of each primer, and 250 nM probe. The thermal cycling conditions included 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min for denaturing and anneal extension, respectively. For relative quantification of the expression of IL-25 mRNA, primers 5′CGGAGGAGTGGCTGAAGTGGAG and 5′ATGGG TACCTTCCTCGCCATG were used in a reaction mix containing 20 ng (cell line cDNA libraries) or 50 ng (whole tissues) cDNA with a reporter fluorescent dye, SYBR. PCR amplification of the housekeeping gene ubiquitin was performed for each sample to control for sample loading and to allow normalization between samples according to the manufacturer's instructions (Perkin Elmer). Sense and antisense primers, as well as probes used for the detection of all other genes of interest, were predeveloped TaqMan assay reagents (Perkin Elmer). Primers and probes were designed to ensure that no cross-reactivity with other genes would occur. The probes for each message were labeled at the 5′ end with a reporter fluorescent dye, FAM, and at the 3′ end with a quench fluorescent dye, TAMRA. Fluorescence detection of FAM was performed at the end of each cycle. The quantity of cDNA of the gene of interest was directly related to the amount of FAM detected after 40 cycles. For quantification of the expression of various cytokine and chemokine mRNA, cDNA plasmids containing the gene of interest were used as a standard curve, ranging from 100 to 0.01 pg. From this standard curve, the amount of cDNA of the gene of interest was calculated in fg/50 ng total cDNA. As internal control, 18S rRNA expression was measured in each sample in a multiplex assay; the probe for rRNA was labeled at the 5′ end with the reporter fluorescent dye VIC. The amount of 18S rRNA was correlated with the cycle at which VIC fluorescence was first detected (cycle threshold value). In order to correct for any variation in the amount of RNA between individual samples, the mean cycle threshold value for 18S rRNA was calculated for all samples, subtracted from each individual cycle threshold value, and then this difference was raised to the second power and multiplied by the FAM value (in fg/50 ng total cDNA) for each sample. Thus the amount of cDNA of the gene of interest in each sample could be directly compared to amounts detected in all other samples.

Mice and In Vivo Treatments

C57BL/6, BALB/c, and 129 SvEv wild-type (WT) mice were obtained from Taconic Farms (Germantown, NY) or from a colony maintained at the DNAX Animal Care Facility. C57BL/6 RAG1-deficient and 129SvEv RAG2-deficient (RAG KO) mice were obtained from either Jackson (Bar Harbor, ME), Taconic Farms, or from a colony maintained at the DNAX Animal Care Facility. IL-4 KO mice (Kühn et al., 1991) were backcrossed for 10 generations onto the BALB/c background. IL-4Rα KO BALB/c mice were a generous gift from Dr. Frank Brombacher (Noben-Trauth et al., 1997). IL-13 KO 129 Sv/Ev mice were from a colony maintained at the DNAX Animal Care Facility (McKenzie et al., 1998). Mice were given i.p. 0.1 to 10 μg IL-25 protein daily for up to 10 days. For adenovirus infection, mice were given 5×10^{10} virus particles intravenously in the tail vein. For co-treatment with mAb, mice were given i.p. 2 mg of either isotype control mAb or anti-IL-5 (TRFK5) mAb 2 days before the start of IL-25 protein injections (5 μg/day) and were given another 1 mg of mAb 7 days later.

Blood and Spleen Cell Analyses

Blood was collected in micropipettes, diluted in Haema-Line 2 silos (BioChem ImmunoSystems, Allentown, PA), and run on a blood analyzer, System 9010 CP (Serono-Baker Diagnostics, Allentown, PA), which gives the total white blood cell count per mouse. Blood smears were made concurrently. Individual mouse spleens were made into single-cell suspension, counted by hemocytometer, and then an aliquot was used for a cytospin. Both splenic cytospins and blood smears were stained with Wright's stain and modified Giemsa stain (Sigma Diagnostics, St. Louis, MO) and counted to determine relative percentage of mononuclear cells, neutrophils, and eosinophils. Absolute numbers of each cell type were then calculated for individual mice.

Determination of Serum Ig Isotypes

Serum Ig was determined either after 10 days of treatment with saline or IL-25 protein or 14 days after infection with Ad-control or Ad-IL-25. IgA from intestinal contents (fecal matter) was determined after 20 days of treatment with either saline or 5 μg/day IL-25 protein. IgG_1 and IgG_{2a} were determined using specific capture and detection antibody pairs (Southern Biotechnology Associates, Birmingham, AL), followed by incubation with streptavidin-horse peroxidase (Jackson ImmunoResearch Labs, West Grove, PA). IgA was captured with rat anti-mouse IgA (PharMingen, San Diego, CA) and detected with NIP-conjugated 2740C. IgE levels were determined using the antibody pairs EM95 and NIP-conjugated 210E. IgA and IgE plates were then incubated with J4-anti-NIP peroxidase. The peroxidase reaction was developed with TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and stopped with 1 M phosphoric acid. Plates were read on a plate reader at 450 nm.

In Vitro Cytokine Production Assay

Single-cell suspension was made from C57BL/6 WT (Figure 5B, upper panel), C57BL/6 RAG 1 KO (unfractionated and depletion studies; Figure 5B, upper and lower panel), and 129 SvEv RAG 2 KO (cell sorting; Figure 5B, lower panel, Figure 5C) spleens. Red blood cells were lysed with a NaCl gradient. To deplete WT splenocytes of T and B cells, cells were incubated with anti-CD4, anti-CD8, and anti-B220 mAb supernatants (10% v/v), then mixed with goat anti-rat IgG (Fc)-coated and goat anti-rat IgG (H+L)-coated magnetic beads (PerSeptive Diagnostics, Cambridge, MA), and the positive cells were removed magnetically. Cells were plated at 10^5 cells per well in triplicate with or without IL-25 in a 96-well U-bottom microtiter plate in RPMI 1640 + 10% heat inactivated fetal bovine serum supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate. Cell-free supernatants were collected after 72 hr and assayed for IL-13 by ELISA. IL-13 was detected using the Quantikine M mouse IL-13 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN) in accordance with the manufacturer's instructions. To deplete RAG splenocytes of granulocytes and myeloid cells, the cells were incubated with anti-CD4, anti-B220, anti-Gr-1, and anti-CD11b mAb supernatants, positive cells were removed

using magnetic beads, and the remaining cells were cultured as described above. For sorting cells based on MHC class II expression, spleens were taken from RAG KO mice that had been treated for 4–5 days with 5 μg IL-25. The splenocytes were stained with anti-I-A^b and anti-CD11c mAbs (both from PharMingen), sorted on a FACS Vantage SE (Becton-Dickinson, Mountain View, CA) based on their MHC class II expression (>95% purity), and then cultured as described above. After sorting, some MHC class II^{high} cells were further stained with anti-CD8α mAb (PharMingen) for further FACS analysis.

Histologic Analyses

Microscopic examination of mouse tissues was performed in a blinded fashion by the same pathologist (M.W. Leach) on formalin-fixed tissue sections stained with either hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), or alcian blue.

Statistics

All data were analyzed using a statistical program (InstatP). A Student's t test or a nonparametric Mann-Whitney test was used to determine statistical significance between groups, with $p \leq 0.05$ being considered significant.

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