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IL-4 Induces Characteristic Th2 Responses Even in the Combined Absence of IL-5, IL-9, and IL-13

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Summary

Functional redundancy is highly prevalent among the Th2 interleukins (IL)-4, IL-5, IL-9, and IL-13. To define the critical functions of these cytokines, we have generated a novel panel of compound Th2 cytokine-deficient mice (from single to quadruple cytokine knockouts). We find that these Th2 cytokines are not essential for fetal survival even during allogeneic pregnancy. Using intestinal parasite infection and a pulmonary granuloma model, we demonstrate cryptic roles for IL-4, IL-5, IL-9, and IL-13 in these responses. Significantly, although IL-5, IL-9, and IL-13 add to the speed and magnitude of the response, a threshold is reached at which IL-4 alone can activate all Th2 effector functions. These mice reveal distinct spatial, temporal, and hierarchical cytokine requirements in immune function.

Introduction

As the immune system evolved under selective pressure from pathogen challenge, a range of genes with diverse and overlapping effector functions has emerged. This redundancy in function of distinct genes has proven a confounding factor in the analysis of many knockout mouse lines. Often individual gene deletion leads to no discernible phenotype or a biological effect that is only observed by further ablation of other family members. One system that contains a high degree of compensatory pathways is the network of cytokines secreted during immune reactions. Indeed, the T cell-derived cytokines such as IL-4, IL-5, IL-9, and IL-13 have all been implicated in diseases such as asthma and allergy, parasite infection, and autoimmunity, but their relative importance in specific disease states often remains obscure due to functional redundancy (Finkelman et al., 1997; Holgate, 1999). Consequently, therapeutic strategies based on blocking individual Th2 cytokine functions, for example antagonism of IL-5 in asthma, have proven ineffective (Leckie et al., 2000). Therefore, to rationally design successful therapeutic interventions based on these cytokines, it is essential that we understand the relative importance of each interleukin in immune reactions and also their functional redundancy.

Over the last two decades, the identification of T cell subsets defined by their cytokine expression profiles has become a central paradigm for the classification of immune responses. Thus, T helper 1 (Th1) responses are defined by the expression of interleukin-2, lymphotoxin-β, and interferon-γ and are characteristic of delayed-type hypersensitivity responses and cell-mediated immune reactions to inflammatory stimuli such as bacteria, viruses, intracellular parasites, and inappropriate autoimmunity. In contrast, T helper 2 (Th2) responses are defined by the production of IL-4, IL-5, IL-9, and IL-13 and are typical of humoral immune reactions, helminth infection, and atopic diseases such as asthma. This model has also been extended to accommodate cell lineages that reinforce the differential cytokine profile, with Th1 and Th2 being referred to as type 1 and type 2 responses, respectively. Defining the interrelationship of the Th1 and Th2 responses and their relative impact on the progression of immune-mediated pathology remains a central theme of immunology.

The complex interactions of the cytokine network make understanding its function extremely challenging. Toward this understanding, significant progress has been made by studying the individual Th2 cytokines either in vitro or using conventional transgenic mice or gene-targeted mice (Kopf et al., 1996; Kuhn et al., 1991; McKenzie et al., 1998b; Townsend et al., 2000). However, due to the complexity of the network, compensatory mechanisms have been reported during the induction of cytokine-regulated immune activation. Collectively, studies relying on the deletion of individual Th2 genes have illustrated that immune responses, which are often pathological if uncontrolled, are not regulated solely by the product of a single gene.

In order to unravel the labyrinthine complexity of the Th2 response, we have used multiple rounds of gene targeting to generate a novel panel of Th2 cytokine-deficient mice. Using this combinatorial gene disruption strategy, we have generated mouse lines with compound deficiencies in one, two, three, or four Th2 cytokines. The production of these animals has enabled us to initiate studies to demarcate the roles of IL-4, IL-5, IL-9, and IL-13 in specific immune responses and to elucidate the compensatory cytokine-mediated pathways that are inherent in the regulation of Th2 immune responses. The immune function of transgenic mice was evaluated in two distinct Th2-modulated in vivo models: challenge with the gastrointestinal nematode parasite *Nippostrongylus brasiliensis* (Finkelman et al., 1997) and the synchronous Th2 and Th1 pulmonary granuloma models (Chensue et al., 1995).

Results and Discussion

Generation of Th2 Cytokine-Deficient Mice

The genes encoding the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 all map to a gene cluster on human chromo-

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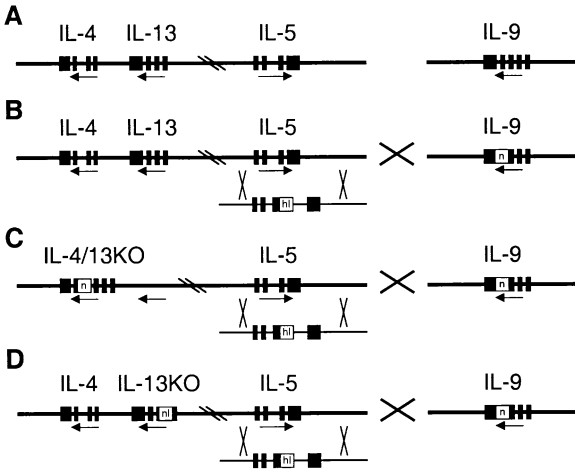

Figure 1. Cytokine Cluster and Gene Disruption Strategy

(A), The structure of the cytokine locus; (B), disruption of the IL-5 locus; (C), disruption of the IL-5 locus in ES cells carrying a disruption of the IL-4 and IL-13 genes; (D), disruption of the IL-5 locus in ES cells carrying a disruption of the IL-13 gene. Animals derived from ES cells carrying the predicted homologous recombination events were then crossed with IL-9KO mice. n, neomycin resistance cassette; nl, neomycin and lacZ cassette; hl, hygromycin and lacZ cassette.

some 5q (Frazer et al., 1997), with a similar gene linkage observed on mouse chromosome 11, where *il-4*, *il-5*, and *il-13* are located (McKenzie et al., 1993), with *il-9* mapping to mouse chromosome 13 (Mock et al., 1990). Due to this juxtaposition, it is unlikely that crossover events would occur at high frequency between these loci during intercrossing of mice carrying a disruption of the individual cytokine genes. Therefore, we have undertaken multiple rounds of gene targeting in embryonic stem (ES) cells to generate mouse lines containing combinations of disruptions in the *il-4/il-5/il-13* gene cluster. Animals derived from ES cells targeted at multiple alleles were then interbred with IL-9-deficient animals to complete the disruption of up to four Th2 cytokines (Figure 1 and Table 1). We have used these animals to investigate the functional redundancy and compensatory roles of these cytokines in responses to intestinal parasite infection and pulmonary antigen challenge.

Absence of Four Th2 Cytokines Does Not Reduce Fetal or Neonatal Survival Even in Allogeneic Pregnancy

The nonrejection of the fetus still remains an immunological enigma despite the elucidation of several mechanisms that appear to facilitate embryo survival, including downregulation of major histocompatibility (MHC) expression in the placenta and inactivation of maternal T cells by tryptophan deprivation (reviewed by Mellor and Munn, 2000). The presence of Th2 cytokines at the maternal-fetal interface has also been proposed as a mechanism for the privileged survival of the fetus despite the surveillance of the maternal immune system and the presence of paternal MHC antigens (Wegmann et al., 1993). However, few direct experiments have been undertaken to determine the critical requirement for Th2 cytokines during pregnancy, and the theory remains controversial. It has been suggested that the expression of the Th2 cytokines is necessary for counteracting the potentially detrimental effects of the proinflammatory type 1 response (Piccinni et al., 2000). For example, while IL-4, IL-5, and IL-10 are detectable at the maternal-fetal interface throughout murine gestation, IFN-γ is only expressed early and transiently during pregnancy (Lin et al., 1993). Thus, it might have been anticipated that by removing even more of the Th2 cytokines the survival of the fetus would have been compromised. However, normal Mendelian distribution of offspring genotypes was detected from all the heterozygous breeding pairs despite the ablation of up to four Th2 cytokines, indicating that homozygous null fetuses did not require these Th2 cytokines for normal gestation (data not shown). Mating of homozygous null mice also gave rise to normal litter sizes (Table 1), and all homozygous offspring from the multiple cytokine deficient mouse lines were healthy. In these breeding programs, all the animals were of the 129 $\times$ C57BL/6 (F2) background with matched MHC antigens (H-2b). We next tested whether multiple Th2 cytokine gene disruption would influence fetal survival in the presence of disparate MHC genes. To test if maternal Th2 cytokine production was necessary for fetal survival, male BALB/c (H-2d) mice were set up with female IL-4/5/9/13KO 129 $\times$ C57BL/6 (H-2b) mice. These cytokine-deficient females gave birth to litters of equivalent size to those derived from wild-type breeders (BALB/c crossed with 129 $\times$ C57BL/6) and from breeders in which the males were Th2 cytokine deficient and the females were wild-type (IL-4/5/13KO BALB/c (H-2d) crossed with 129 $\times$ C57BL/6) (Table 1). These data indicate that successful allogeneic pregnancy is not dependent on Th2 cytokines expressed by the mother. In a further experiment, male IL-4/5/13KO BALB/c (H-2d) mice were crossed with female IL-4/5/9/13KO 129 $\times$ C57BL/6 (H-2b) mice. We found that litter sizes did not differ from those of the other breeding experiments outlined above (Table 1), indicating that even in MHC-mismatched animals the absence of IL-4, IL-5, and IL-13 in the fetus and IL-4, IL-5, IL-9, and IL-13 in the mother did not impair pregnancy. Thus, the expression of these Th2 cytokines at the maternal/fetal interface is not obligatory for normal gestation. However, further studies will be necessary to determine whether the additional disruption of IL-10 may alter fetal survival.

Compound Cytokine-Deficient Mice Develop Normally

Despite the combined disruption of four immunoregulatory cytokines, T cell numbers and lymphoid organ composition were overtly normal, as determined by flow cytometric analysis using a panel of antibodies specific for hematopoietic cell surface markers (Townsend et al., 2000) (data not shown).

Delineation of Th2 Cytokine Functional Redundancy Using *N. brasiliensis* Infection

Despite the long-recognized connection between infection with the parasitic worm *N. brasiliensis* and the induction of Th2 cytokine production, the relative importance of the different cytokines to the immune response

Table 1. Syngeneic Nor Allogeneic Intercrossing of Compound Th2 Cytokine-Deficient Mice Influences Litter Survival

Genotype of Male × Female Cross (Syngeneic Intercross)	Mean Litter Size at Weaning (n = 13)
Wild-type 129/C57BL/6 (F2) intercross	5.8
Single gene deletions	
IL-4KO C57BL/6 intercross (Kuhn et al., 1991)	6.76
IL-5KO 129/C57BL/6 (F2) intercross	6.15
IL-9KO 129/C57BL/6 (F2) intercross (Townsend et al., 2000)	6.8
IL-13KO 129/C57BL/6 (F2) intercross (McKenzie et al., 1998a)	6.8
Double gene deletions	
IL-4/9KO 129/C57BL/6 (F2) intercross	viable[a]
IL-4/13KO 129/C57BL/6 (F2) intercross (McKenzie et al., 1999)	6.9
IL-5/9KO 129/C57BL/6 (F2) intercross	6.54
IL-5/13KO 129/C57BL/6 (F2) intercross	7.6
IL-9/13KO 129/C57BL/6 (F2) intercross	viable[a]
Triple gene deletions	
IL-4/5/13KO 129/C57BL/6 (F2) intercross	6.86
IL-4/9/13KO 129/C57BL/6 (F2) intercross	viable[a]
IL-5/9/13KO 129/C57BL/6 (F2) intercross	6.38
Quadruple gene deletions	
IL-4/5/9/13KO 129/C57BL/6 (F2) intercross	7.15
Genotype of Male × Female Cross (Allogeneic Intercross)	**Mean Litter Size at Weaning (n = 3)**
BALB/c × 129/C57BL/6 (F2)	9.3
IL-4/5/13KO BALB/c × 129/C57BL/6 (F2)	8.3
BALB/c × IL-4/5/9/13KO 129/C57BL/6 (F2)	8.6
IL-4/5/13KO BALB/c × IL-4/5/9/13KO 129/C57BL/6 (F2)	10

[a] Insufficient litter numbers to include data.

has been difficult to attribute due to the apparent functional redundancy of the Th2 response. We have shown previously that IL-13 plays a critical role in the effective expulsion of the gastrointestinal helminth parasite *N. brasiliensis*. However, although IL-13 was found to be essential for rapid parasite expulsion, worms continued to be expelled, but with slower kinetics than those in wild-type mice (McKenzie et al., 1998a). Furthermore, although deletion of IL-4 alone did not retard worm expulsion, we observed that its combined deletion with IL-13 resulted in an additional impairment to worm expulsion (McKenzie et al., 1999). At that time, we noted that even though IL-4 and IL-13 played additive roles in helminth immunity, worms continued to be expelled, albeit more slowly, in their absence. These studies began to unearth some of the hidden redundancies of the Th2 cytokine response, but also highlighted the complexity of cytokine-mediated immunoregulation and suggested that other factors were also able to play compensatory roles. However, data from individual cytokine knockout mice, IL-4 (Finkelman et al., 1997; Lawrence et al., 1996), IL-5 (Kopf et al., 1996), and IL-9 (Townsend et al., 2000), failed to indicate that the other Th2 cytokines impacted on this response. In contrast, overexpression of IL-4, IL-5, and IL-9 in transgenic mice or by using recombinant cytokine protein has been reported to enhance parasitic worm expulsion (Dent et al., 1999; Faulkner et al., 1997; Finkelman et al., 1997). It is, perhaps, surprising that despite decades of research, the cytokine regulation of parasitic worm expulsion, most notably in the case of the extensively studied *N. brasiliensis*, remains obscure.

IL-5 Plays an Additive Role in Worm Expulsion

In previous experiments, we have demonstrated that while wild-type mice expel *N. brasiliensis* within 5–10 days post-infection, the deletion of IL-13 or IL-4/13 impairs expulsion by up to 2- to 3-fold (McKenzie et al., 1998a, 1999). However, although expulsion was delayed, worms continued to be expelled. One possible reason for the continued expulsion was the elevated level of IL-5 and the accompanying eosinophilia observed in the IL-4/13KO mice. To address whether IL-5 could also play a role in *N. brasiliensis* expulsion, we generated animals which lacked IL-4, IL-5, and IL-13 (IL-4/5/13KO) and assessed their ability to expel parasitic worm infection. As expected, wild-type animals expelled all worms from their intestines by day 10 post-infection, while worm expulsion from the intestines of the IL-4/13KO animals was considerably delayed, with almost half of the worms remaining in the intestine after 25 days (Figure 2A). When we compared the expulsion of parasites from the intestines of IL-4/5/13KO animals, we found that the additional deletion of IL-5 resulted in a further delay in worm clearance, with 50% worm expulsion at day 35 (Figure 2A). Thus, while previous studies failed to show a role for IL-5 deletion in worm expulsion, the compound absence of IL-5 with IL-4 and IL-13 significantly delays worm expulsion, with more worms surviving in the IL-4/5/13KO animals when compared to IL-4/13KO mice (at day 30, $p = 0.0026$).

IL-9 Plays an Additive Role in Worm Expulsion

We were somewhat surprised to observe that even in the compound absence of IL-4, IL-5, and IL-13, infected mice continued to expel *N. brasiliensis*. We had found previously that IL-9-deficient mice expelled *N. brasiliensis* with similar kinetics to those observed for wild-type mice; however, we also noted that following infection, production of other Th2 cytokines was enhanced in the IL-9-deficient mice, suggesting that compensatory cytokine expression might be occurring (Townsend et

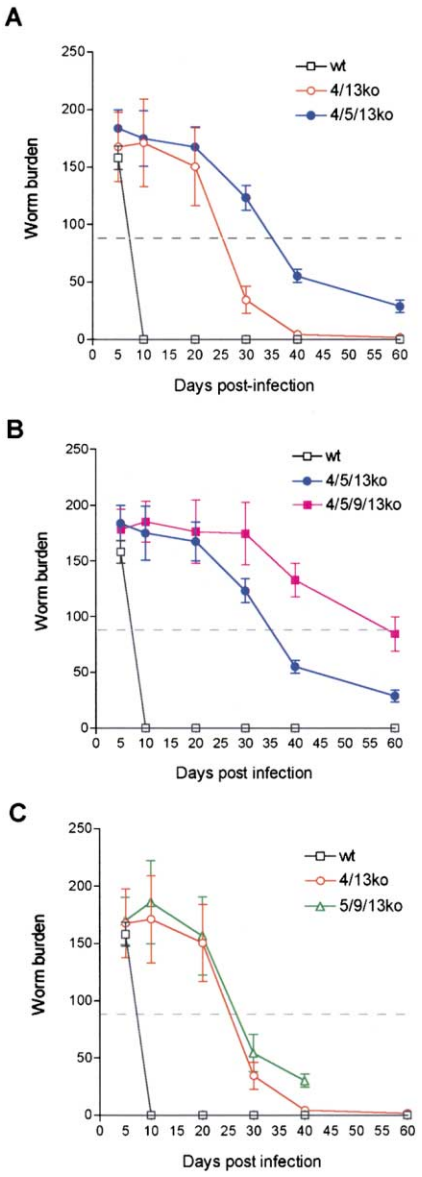

Figure 2. Analysis of Infection of Wild-Type and Compound Cytokine-Deficient Mice with *N. brasiliensis*

Cohorts of 4–6 infected mice were sacrificed at the times indicated to obtain intestinal worm counts. Data are representative of two repeat experiments. Data are presented as means plus SEM. (A), Determination of worm burdens from wild-type, IL-4/13KO, and IL4/5/13KO mice; (B), determination of worm burdens from wild-type, IL-4/5/13KO, and IL4/5/9/13KO mice; (C), determination of worm burdens from wild-type, IL-4/13KO, and IL-5/9/13KO mice.

al., 2000). To test whether IL-9 plays a hidden role in worm expulsion, we crossed the IL-4/5/13KO mice with IL-9KO mice to generate IL-4/5/9/13KO animals. These animals were then tested for expulsion of *N. brasiliensis* infections. The additional deletion of IL-9 resulted in a further delay in the expulsion of parasites from the intestine (Figure 2B). Indeed, the compound deletion of IL-4, IL-5, IL-9, and IL-13 leads to a delay in expulsion of over one order of magnitude, with almost half of the worms still present in the intestines of IL-4/5/9/13KO mice after 60 days. From 40 days post-infection onward, significantly more worms were present in the IL-4/5/9/13KO animals than in the IL-4/5/13KO mice (at day 40, $p = 0.0028$; at day 60, $p = 0.0183$). These experiments highlight the additive roles of the four Th2 cytokines in the clearance of helminth parasites and further demonstrate the value of using compound Th2 cytokine knockouts.

IL-4 Can Compensate for IL-5, IL-9, and IL-13 in Worm Expulsion

The deletion of IL-4 in mouse knockout lines has provided an important tool for understanding the fundamental roles of this cytokine in immune responses (Kopf et al., 1993; Kuhn et al., 1991). However, since IL-4 is a critical component in the normal generation of Th2 cells, its deletion results in a phenotype that is characterized by the impaired expression of IL-5, IL-9, and IL-13 (Kopf et al., 1993). Thus, the downstream consequence of IL-4 disruption may have a direct outcome on its effector functions (in addition to Th2 differentiation) or an indirect effect resulting from a reduction in cytokine expression as a consequence of fewer Th2 cells. To address the functional importance of IL-4, we have assessed its potency in the compound absence of IL-5, IL-9, and IL-13. Following infection with *N. brasiliensis*, IL-5/9/13KO animals were observed to have retarded worm expulsion similar to that observed in the IL-4/13KO mice (Figure 2C) but were still able to induce worm expulsion. Thus, even in the absence of the other Th2 cytokines, IL-4 alone induces worm expulsion, indicating that IL-4 can initiate worm clearance independently of its role in enhancing Th2 cytokine expression.

Cytokine Production during *N. brasiliensis* Infection

As expected, the wild-type cytokine response to *N. brasiliensis* infection was dominated by the Th2 cytokines IL-4, IL-9, IL-5, and IL-13 and the Th2-related cytokine IL-10 (Figure 3). Two striking effects on cytokine expression were observed in the compound cytokine-deficient mice. First, only in the combined absence of IL-4, IL-5, IL-9, and IL-13, where worm expulsion was most severely impaired, did the immune response divert to a Th1 response characterized by IL-12 expression (Figure 3). Interestingly, IL-4 was not the sole factor limiting this redirection of the response, since neither IL-5/9/13KO mice (IL-4 present) nor IL-4/13KO (IL-4 absent) mice switched toward an IL-12 dominated Th1 response. These data indicate that while IL-4 plays a dominant role in promoting the Th2 response, the biological functions of the other Th2 factors also act to perpetuate the response. Secondly, in the absence of IL-5, IL-9, and IL-13 (IL-5/9/13KO mice), IL-4 expression was greatly enhanced, with levels 4- to 5-fold greater than those in wild-types at days 5, 10, and 20 post-infection (Figure 3). Thus, in the absence of the other Th2 cytokines IL-4 expression was upregulated in an attempt to compensate for Th2 cytokine deficit. Interestingly, these elevated levels of IL-4 correlated with profoundly enhanced expression of IL-10. Comparison of the IL-10 expression by the IL-5/9/13KO mice with the IL-10 production by the IL-4/5/9/13KO mice demonstrated that IL-4 was directly responsible for this induction of IL-10 (Figure 3). It is

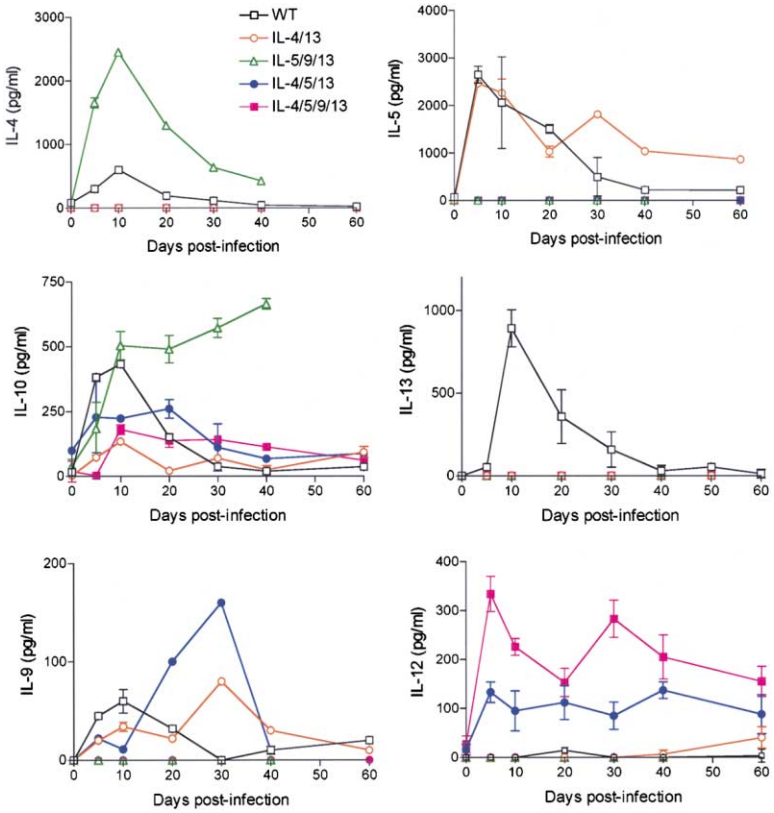

Figure 3. Analysis of Cytokine Expression in Response to *N. brasiliensis* Infection

Following infection with *N. brasiliensis*, mesenteric lymph node cells from wild-type and compound cytokine-deficient animals were stimulated with *N. brasiliensis*-antigen (NbES). Supernatants were analyzed by cytokine ELISA. Data are presented as means plus SEM. Cohorts of 4–6 mice were infected with 500 viable third-stage *N. brasiliensis* larvae. Data are representative of two repeat experiments.

currently unclear what role IL-10 is playing in *N. brasiliensis* expulsion. However, given the role of IL-10 in mast cell development (Thompson-Snipes et al., 1991) and its recently reported role in immunity to *Trichuris muris* (Schopf et al., 2002), it is possible that this cytokine may impact on *N. brasiliensis* clearance.

IL-9 Induces Mastocytosis in the Absence of IL-4, IL-5, and IL-13 during *N. brasiliensis* Infection

During parasite infection in wild-type mice, extensive mast cell hyperplasia occurs in the intestinal mucosa, and this is associated with parasite expulsion. Several cytokines can modulate mast cell development, and it is unclear which are critical. To 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 wild-type and cytokine-deficient mice. Wild-type mice initiated a rapid mast cell response to infection, and this correlated with worm clearance (Figure 4A). Significantly, compound cytokine-deficient mice lacking IL-4, IL-5, and IL-13 continued to generate mast cell hyperplasia equivalent to wild-type (Figure 4A). However, the additional disruption of IL-9, in the compound IL-4/5/9/13KO, completely ablated this response (Figure 4A). Thus, using compound cytokine-deficient mice we have now demonstrated that IL-9 can play a key role in the generation of the mastocytosis induced by *N. brasiliensis* infection and that this effector function is normally eclipsed by an additional factor. Examination of the levels of mMCP-1 found in the compound IL-5/9/13KO animals following *N. brasiliensis* infection clearly illustrates that IL-4 is the compensatory factor for IL-9 in the generation of mastocytosis (Figure 4A). Histological analysis of toluidine blue-stained mast cells demonstrated that mMCP-1 expression correlated with jejunal mast cell numbers (data not shown). Thus, only in the combined absence of IL-4 and IL-9 do intestinal mast cells fail to expand in response to infection. Additional studies will be necessary to determine whether this observed modulation of mastocytosis is dependent on other regulators of mast cell development, including stem cell factor and IL-3 (Galli and Hammel, 1994; Madden et al., 1991).

Cytokine Regulation of IgE during *N. brasiliensis* Infection

Upregulation of IgE is characteristic of the immune response to intestinal helminth infections. However, no obligate role has been identified for IgE in the process of worm expulsion (Finkelman et al., 1997). IL-4 and IL-13 have both been shown to play roles in the regulation of IgE production (Kuhn et al., 1991; McKenzie et al., 1998b). Following infection of wild-type mice with *N. brasiliensis*, levels of IgE rose significantly before peaking at around day 20 post-infection and falling back to baseline by day 40 post-infection (Figure 4B). As expected, those mouse lines lacking IL-4 failed to produce IgE (Figure 4B). In contrast, the IL-5/9/13KO mice rapidly initiated IgE production, and levels of IgE were still rising at the termination of the experiment at day 40 post-infection (Figure 4B). Thus, in the absence of IL-5, IL-13, and IL-9 the immune system compensates, via IL-4, with alternative effector functions from the Th2 repertoire.

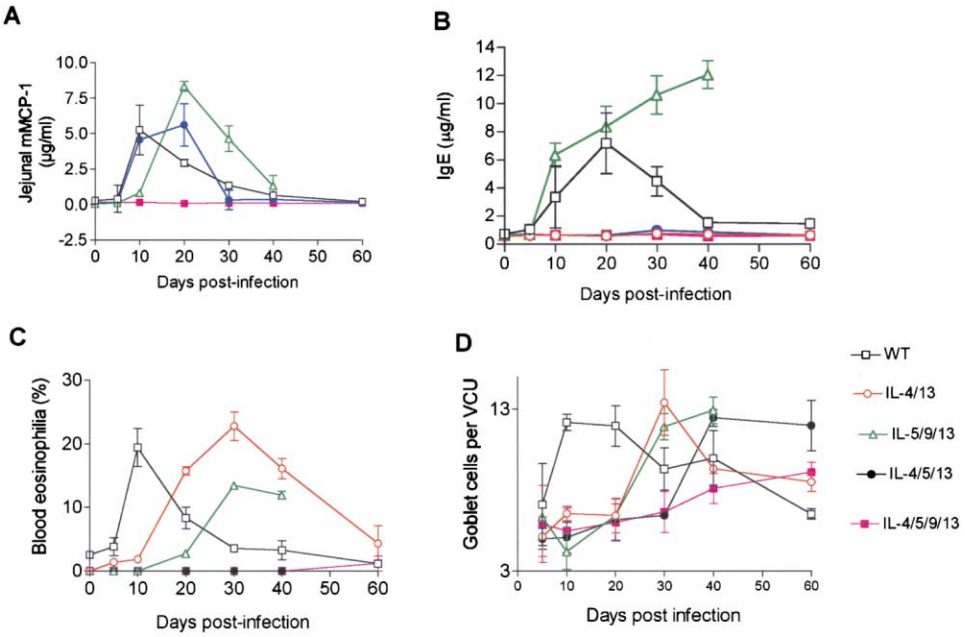

Figure 4. Analyses of Mast Cell Protease-1, IgE, Eosinophilia, and Goblet Cell Hyperplasia in Response to *N. brasiliensis* Infection

Data are presented as means plus SEM. Representative data are from two repeat experiments using 4–6 animals per group.

(A) Mouse mast cell protease-1 (mMCP-1) activity was measured from jejunal homogenates. Data are expressed as micrograms mMCP-1 per milligram of jejunal protein.

(B) Total serum IgE expression from wild-type and compound cytokine-deficient mice following infection.

(C) Blood eosinophilia after *N. brasiliensis* infection.

(D) Determination of goblet cell number per villus crypt unit (VCU).

IL-4 Induces IL-5-Independent Eosinophilia during *N. brasiliensis* Infection

Eosinophilia is also characteristic of Th2 responses induced by helminth infection. Significant evidence links the expression of IL-5 with the induction of eosinophilia (Sanderson, 1992), and IL-5KO and IL-5-receptorKO mice produce only basal numbers of eosinophils (Kopf et al., 1996; Yoshida et al., 1996), while overexpression of IL-5 elicits profound eosinophilia (Dent et al., 1990). Upon infection with *N. brasiliensis*, wild-type mice developed eosinophilia by day 10 post-infection (Figure 4C), correlating with expression of IL-5. As we have shown previously, eosinophilia was delayed in the IL-4/13KO following infection, but once induced the eosinophilia correlated with worm expulsion (Figure 4C) (McKenzie et al., 1999). To test if the additional deletion of IL-5 altered the induction of eosinophilia, we examined eosinophil numbers following infection. Only in the combined absence of IL-4, IL-5, and IL-13 was the induction of eosinophilia completely blocked (Figure 4C), and this correlated with the additional delay in worm expulsion observed in this mouse line (Figure 2A). Despite the purported role for IL-9 in eosinophilia, this cytokine cannot compensate for this response when the other Th2 cytokines are absent. However, it should be noted that this may not be the case for all intestinal parasite species where a role for IL-9 in eosinophilia has been suggested (Richard et al., 2000). Given the predominant role played by IL-5 in generating eosinophilia, we were struck by the profound eosinophilia induced by IL-4 even in the absence of IL-5, IL-9, and IL-13. The role of IL-4 in this process was proven by the absence of eosinophilia in the IL-4/5/9/13KO line (Figure 4C). Thus, we demonstrate for the first time that IL-4 is capable of inducing IL-5-independent eosinophilia. Further studies will be necessary to determine whether IL-4 acts directly on eosinophils in this response or via cytokines such as IL-3, GM-CSF, or eotaxin, all of which can alter eosinophil development.

IL-4, IL-5, IL-9, and IL-13 Can All Play Roles in the Goblet Cell Response during *N. brasiliensis* Infection

The production of mucus represents an important response for protecting mucosal surfaces. Although Th2 cell-mediated 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. IL-4 is presumed to be necessary for the generation of IL-9- and IL-13-producing Th2 cells (Cohn et al., 1999; Grunig et al., 1998; Louahed et al., 2000; McKenzie et al., 1998a; Wills-Karp et al., 1998), while IL-9 and IL-13 are believed to directly regulate goblet cell production and function (Cohn et al., 1999; Grunig et al., 1998; Louahed et al., 2000; McKenzie et al., 1998a; Wills-Karp et al., 1998).

Using the compound cytokine-deficient mice, we examined goblet cell hyperplasia over an extended period following *N. brasiliensis* infection. Although ablation of IL-13 has been found to reduce goblet cell numbers following the induction of Th2 responses (McKenzie et

al., 1998a), during our present study we have found that even in the combined absence of IL-4 and IL-13 (IL-4/13KO) goblet cell hyperplasia does occur but is considerably delayed. No increase in goblet cell numbers was detected at day 10 or day 20 post-infection of the IL-4/13KO mice (Figure 4D), but by day 30 goblet cell numbers had reached levels equivalent to those observed in the wild-type mice at day 10 (Figure 4D), and in both cases correlated with worm expulsion (Figure 2A). The additional deletion of IL-5 (IL-4/5/13KO) and IL-9 (IL-4/5/9/13KO) resulted in further impairments in the development of goblet cells during infection (Figure 4D). Increased numbers of goblet cells were eventually observed in both the IL-4/5/13KO lines, and once again their increase correlated with worm expulsion (Figure 2B). In the absence of all four cytokines, goblet cell numbers barely increased. Thus, although IL-13 is considered the major mediator of goblet cell hyperplasia, we now demonstrate an additive effect of all four Th2 cytokines in this process. It is noteworthy that even in the absence of all four Th2 cytokines, the basal numbers of goblet cells remained similar to wild-type, indicating that these cytokines do not play an essential role in the development or homeostasis of this cell population.

Once again, analysis of the IL-5/9/13KO mice demonstrated the potency of IL-4 in the generation of the effector functions of the Th2 response. Even in the absence of IL-9 and IL-13, which have both been demonstrated to play roles in the induction of goblet cells, IL-4 was able to induce a profound goblet cell response, but only after a delay of approximately 20 days.

Response of Th2 Cytokine-Deficient Mice to Pulmonary Challenge

To complement the chronic infection model using helminth parasite challenge, we wished to address how the Th2 response varied between tissues and in response to alternative antigen stimulus. To determine the individual and combined roles of the Th2 cytokines in the generation of a pulmonary Th2 cytokine-mediated inflammatory response, we employed a model system in which synchronous pulmonary granuloma formation is induced around *Schistosoma mansoni* eggs. This model is characterized by a cellular granulomatous response that develops around parasite eggs that lodge in the lung following their intravenous injection into mice. The response can be quantified following either primary or secondary exposure to antigen and is characterized by the high-level polarized expression of Th2 cytokines, eosinophilia, and goblet cell hyperplasia.

Th2 Granuloma Formation and Pulmonary Responses in Th2 Cytokine-Deficient Mice

Following a single exposure to antigen (primary response), we observed that the presence of IL-4 and IL-13 was essential for the generation of primary granulomas (Figure 5A). These data are in agreement with our previously reported experiments, in which secondary granuloma formation was found to require both IL-4 and IL-13 individually to regulate the response and their combined disruption essentially blocked granuloma formation (Figure 5B). Surprisingly, considering the central role of IL-5 in the generation of eosinophils, and with eosinophils being the dominant cell type within the granuloma, the primary granuloma volumes in the IL-5KO animals were equivalent to wild-type (Figure 5A), and eosinophils continued to be recruited to granulomas in the IL-5KO, though at lower levels than wild-type (Figure 5D). Thus, as we observed following *N. brasiliensis* infection, IL-5-independent eosinophilia is readily observed in the lung.

In the secondary granuloma model, the animals were sensitized with egg antigens 2 weeks before being exposed to secondary pulmonary challenge. While the combined effects of IL-4 and IL-13 disruption were clear in the secondary granuloma, it was also evident that IL-5 was important for the magnitude of the secondary granuloma volume (Figures 5B and 5D), and in its absence fewer eosinophils entered the granuloma (Figures 5C and 5D). However, it must be emphasized that considerable eosinophilia still occurs in the absence of IL-5 (Figure 5D). Significantly, even in the absence of IL-5, IL-9, and IL-13, a sizeable granuloma continued to form with a cellular composition similar to that of the wild-type, though of lower magnitude (Figures 5B and 5D). Thus, while IL-5 and IL-13 contribute to the magnitude of the response, the presence of IL-4 alone among these four Th2 cytokines is able to induce a significant and characteristic Th2 granuloma response, including eosinophilia.

The major roles played by IL-4 and IL-13 in the development of this pulmonary Th2 response were typified by the marked affect their absences had on lung mastocytosis and mucus cell expansion, respectively. It was clear that in this pulmonary model, IL-13 fulfils the major role in mucus cell induction (Figure 5E). Furthermore, although the IL-4KO animals displayed a reduction in goblet cell hyperplasia, IL-4, in the absence of IL-5, IL-9, and IL-13 (IL-5/9/13KO), was insufficient to induce goblet cell expansion (Figure 5E). These results differ from those observed following helminth infection, where the other Th2 cytokines were clearly capable of enhancing the goblet cell response. Strikingly, the absence of mastocytosis in the secondary granuloma response correlated with the ablation of IL-4 expression and was not affected significantly by the combined absence of IL-5, IL-9, or IL-13 (Figure 5F). Once again, this contrasts with the compensatory role observed for IL-9 in the mastocytosis induced following parasitic worm infection. These variations in the mast cell and goblet cell responses may be due to several factors, including specific differences in the tissues under study, the prolonged duration of the helminth infection, the dynamic nature of antigen exposure during infection, and the magnitude of antigen exposure.

Collagen deposition also occurs during the response to pulmonary egg challenge. As reported previously (Fallon et al., 2000), our data demonstrate clearly the importance of IL-13 in this process, with reduced collagen correlating with the disruption of IL-13 (Figure 5G). IL-13 is therefore the dominant profibrotic Th2 cytokine.

Regulation of Cytokine Responses Following Pulmonary Challenge

Cytokine release by antigen-stimulated lymph node cells from naive animals was undetectable except in animals in which all of the Th2 cytokines had been dis-

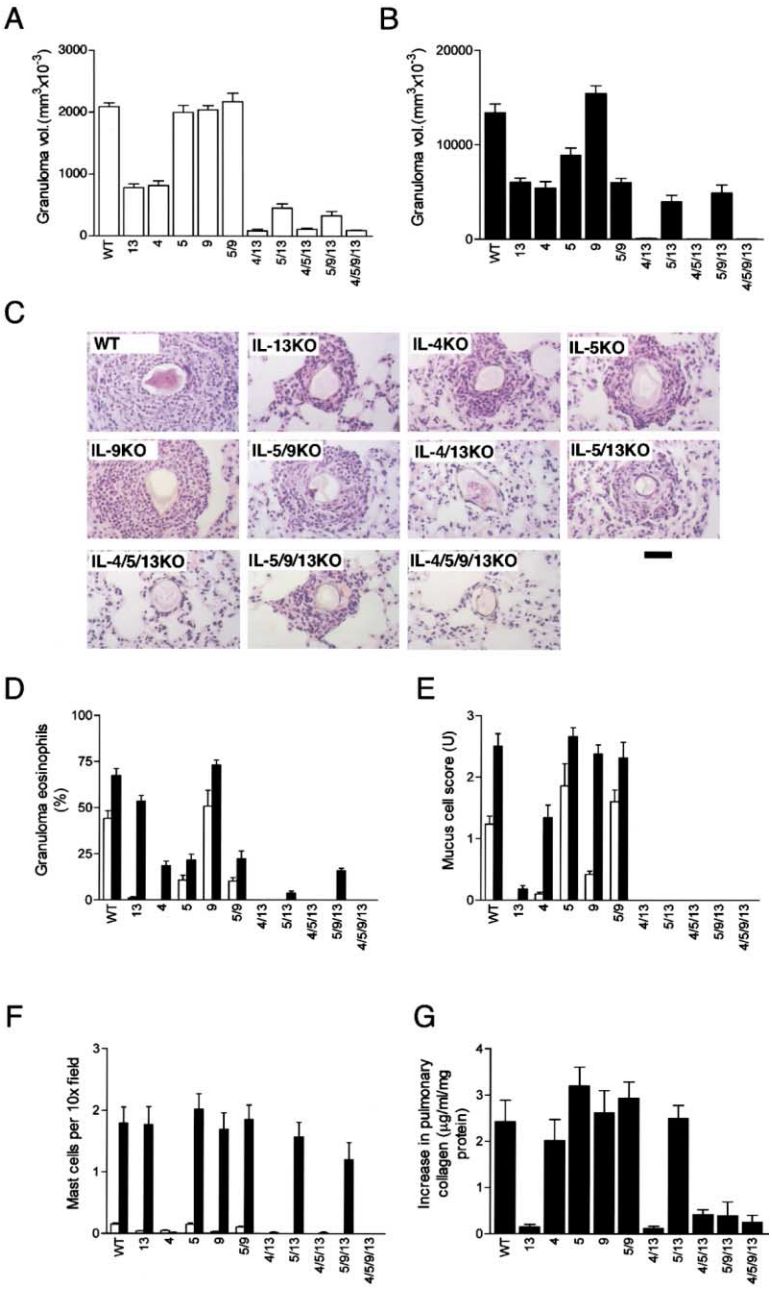

Figure 5. Th2 Granuloma Formation during Pulmonary Th2 Inflammatory Responses

Cohorts of 4–6 mice were used per group. Data are presented as means plus SEM and are representative of two repeat experiments.

(A) Determination of primary granuloma volumes in immunized mice. Mice were injected intravenously with 5000 schistosome eggs to induce synchronous pulmonary granuloma and sacrificed 14 days later.

(B) Determination of secondary granuloma volumes in immunized mice. Mice were injected intraperitoneally with 5000 schistosome eggs and after 14 days challenged intravenously with 5000 schistosome eggs before being sacrificed 14 days later. Lung sections were stained with hematoxylin and eosin, and at least 100 individual granulomas were measured per group.

(C) Histological analysis of individual granulomas. Scale bar, 45 μm.

(D) Granuloma eosinophil counts. Open bars, primary challenge; filled bars, secondary challenge.

(E) Determination of goblet cell number. Open bars, primary challenge; filled bars, secondary challenge.

(F) Mast cell counts. Open bars, primary challenge; filled bars, secondary challenge.

(G) Determination of increase in pulmonary collagen content from Martius scarlet blue-stained lung sections following secondary challenge.

rupted (IL-4/5/9/13KO). In these animals, the background levels of the signature Th1 cytokine IFN-γ were elevated 6-fold (data not shown), indicating that even without antigen activation these cells were biased toward a Th1 profile (naive expression of IL-4, IL-5, IL-9, and IL-13 were below levels of detection; data not shown). This crossregulation between the Th1 and Th2 response was further highlighted following antigen challenge. In the primary and secondary responses, it was clear that the disruption of IL-4 correlated with an increase in the expression of IFN-γ (Figure 6). Unexpectedly, we found that combined disruption of IL-5, IL-9 and IL-13 resulted in the elevated expression of IFN-γ (Figure 6). Examination of IL-4 production revealed a reciprocal pattern of expression to that of IFN-γ (Figure 6), with the IL-5/9/13KO animals expressing lower levels of IL-4 and higher levels of IFN-γ despite retaining a functional IL-4 gene (as evidenced by the normal expression of IL-4 from the IL-5/13KO and IL-5/9KO mice following primary and secondary antigen challenge [Figure 6]). These data demonstrate that the crossregulation of the developing Th1 and Th2 responses requires not only IL-4 for the control of the Th1 arm of the response, but that expression of the other Th2 cytokines is required to sustain this differentiation. It is likely that the reduction of cell lineages normally dependent on IL-5 (eosinophils) and IL-9 (mast cells in the primary response), which would normally release Th2 cytokines including IL-4 (Burd et al., 1995; Sabin et al., 1996), would result in a reduction in cytokine levels. It is clear that the additional

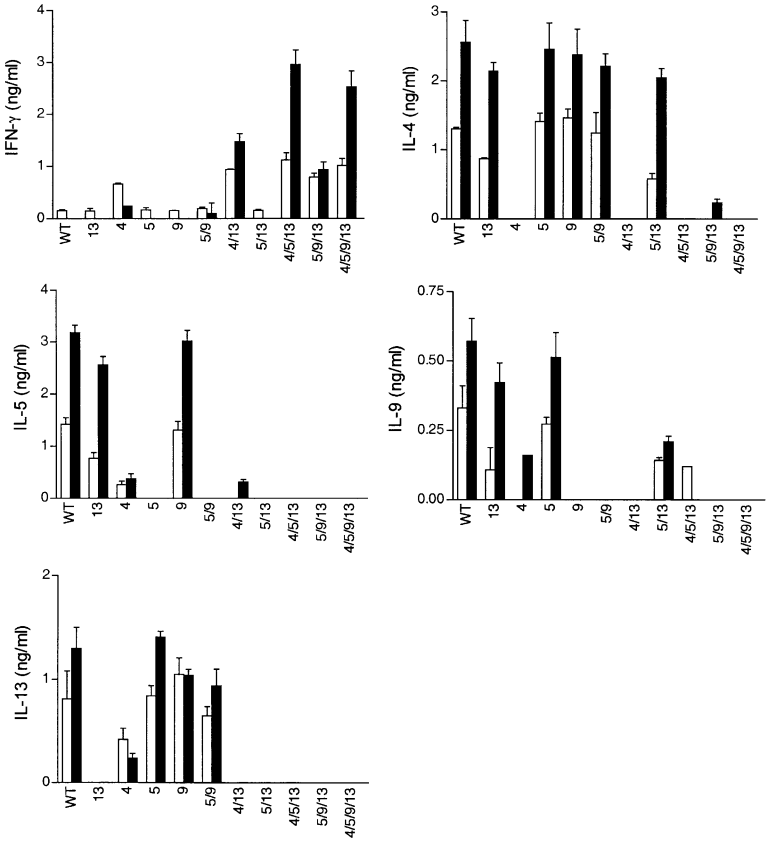

Figure 6. Cytokine Expression during Pulmonary Th2 Inflammatory Responses

Draining mediastinal lymph node cells (5×10^6) from the various mouse lines were cultured with soluble schistosome egg antigens for 3 days, and culture supernatants were assayed for cytokines by ELISA. Data are presented as means plus SD of triplicate wells. Open bars, primary challenge; filled bars, secondary challenge. Data are representative of two repeat experiments.

disruption of IL-13 compounds this deficit, possibly due to its role in suppressing IL-12 release by macrophages, and results in a failure to polarize to a Th2 phenotype (Matthews et al., 2000). Our data demonstrate that if the downstream differentiation and development of the Th2 response, including the activation of effector cells, is impaired, then the response fails to polarize even in the presence of IL-4. Thus, in order to sustain the Th2 response positive feedback from the other Th2 cytokines is required even following secondary immunization. These results support reports in which the overexpression of cytokines such as IL-13 and IL-9 in transgenic mice induces the upregulation of other Th2 cytokines and the exacerbation of Th2 responses (Fallon et al., 2001; Temann et al., 2002). The importance of eosinophil-derived IL-4 has been reported in experiments using IL-5-deficient mice; however, these effects were only evident early (24 hr) in the response (Sabin et al., 1996). In contrast, we see no observable effect in the IL-5KO, but profound synergistic effects following multiple cytokine deletion which persist even following secondary immunization, indicating that this represents an essential pathway that cannot be compensated for by other mechanisms. However, as addressed above, even the reduced levels of IL-4 observed in the IL-5/9/13KO act to induce the development of a significant Th2 granuloma, emphasizing the potency of IL-4 even in the absence of IL-5, IL-9, and IL-13. Once again, these data differ from those recorded in response to intestinal parasite infection, probably due to the magnitude of antigen exposure and the dynamic nature of antigen challenge during infection.

In contrast to the Th2-dominated schistosome egg granuloma, we have also analyzed the response of the Th2 cytokine-deficient mouse lines using a Th1 granuloma model (Chensue et al., 1995). Our results demonstrated a 4-fold enhancement in the magnitude of granuloma formation in the IL-4/13KO and IL-4/5/9/13KO mice characterized by macrophage and neutrophil infiltration and the elevation of the Th1 cytokines IL-12 and IFN-γ (see supplemental data at http://www.immunity.com/cgi/content/full/17/1/7/DC1 for details). These data represent a mirror image of those observed in the Th2-dominated egg granuloma and underscore the reciprocal cytokine regulation that occurs between the Th1 and Th2 responses. It is noteworthy that despite the absence of all four Th2 cytokines that mediate multiple effector functions through several cell lineages, the Th1 response remains constrained, indicating the involvement of other antiinflammatory mediators such as IL-10.

Conclusion

The development of a panel of Th2 cytokine-deficient mice has enabled us to investigate the complex interactions that occur between these molecules in vivo. We have been able, for the first time, to test experimentally the controversial role for Th2 cytokines in pregnancy. Our results demonstrate conclusively that these four cytokines are not essential for the completion of syngeneic or allogeneic pregnancy. We have used a parasitic helminth infection model and a pulmonary inflammation model to identify previously undetectable degrees of cytokine redundancy and compensation. Thus, we have

been able to identify and assign hierarchical effector functions to the Th2 cytokines involved in *N. brasiliensis* expulsion. In the most extreme example, we demonstrate that only in the combined absence of IL-4, IL-5, IL-9, and IL-13 does the Th2 immune response become subverted by Th1 cytokines, and in these animals worm expulsion is very significantly delayed. Our experiments also demonstrate the potency of IL-4. Since IL-4 is required for Th2 cell development and therefore the induction of Th2 cell-produced IL-5, IL-9, and IL-13, it has not been possible to interpret which functions are IL-4 specific and which are dependent on IL-5, IL-9, and IL-13. Even in the absence of IL-5, IL-9, and IL-13, we find that IL-4 induces a potent Th2 response characterized by many of the effector functions for which the other cytokines have biological precedence, including eosinophilia, mastocytosis, and goblet cell hyperplasia. Thus, while IL-5, IL-9, and IL-13 all appear to have primary roles in the rapid induction of effector functions, IL-4 plays a role not only in the differentiation of Th2 cells but also as a key compensatory cytokine for all aspects of the Th2 response. Although our study has concentrated on the effector responses elicited by *N. brasiliensis*, the examination of other nematode infections, such as *Angiostrongylus*, *Trichuris*, and *Strongyloides*, in which IL-5, IL-9, and IL-3 have all been implicated (Finkelman et al., 1997), should also prove informative.

Combinatorial cytokine gene deletion has allowed us to define the functional regulation of the complex effector mechanisms characteristic of the Th2 cell response. Our data demonstrate clearly that these factors act as a coherent interdependent unit critical for the generation of the archetypal Th2 reaction and for the control of the alternative Th1 effector arm. These animals represent an important and novel resource for understanding the compound functions of these cytokines in all aspects of immunobiology and haematopoiesis. They should also prove important for understanding the processes of asthma, allergy, helminth infection, and autoimmunity and thereby facilitate the development of new therapeutic strategies.

Experimental Procedures

Targeted Disruption of the Mouse Interleukin Genes in ES Cells

The IL-5 gene was targeted as follows. A replacement vector was constructed to insert a NotI/SalI cassette containing the following elements (in 5′ to 3′ order): an SpeI linker containing stop codons in all three frames, a retroviral internal ribosomal entry site (IRES) element fused to the lacZ reporter gene with a 3′ polyadenylation signal, and the hygromycin gene into the NotI and XhoI sites engineered at the XmnI site in exon 3 of the IL-5 gene. The targeting vector consisted of 3.5 kb of the IL-5 gene providing the 5′ arm of homology and 2.5 kb comprising the 3′ homology arm. The targeting vector was linearized and electroporated into E14.1 ES cells. To generate IL-4/5/13-deficient mice, ES cells in which the IL-4/13 genes had been targeted previously (McKenzie et al., 1999) were subsequently targeted using the IL-5 gene targeting construct described above. We also used the IL-5 gene targeting construct described above to target ES cells in which the IL-13 gene had been targeted previously (McKenzie et al., 1998a) in order to generate IL-5/13-deficient mice. HindIII-digested genomic DNAs from isolated clones were screened by hybridization using a flanking probe. The probe was made by PCR using the primers 5′-TACAGCTCTCC CTCAGCAGTC-3′and 5′-GCAGCTGGTCTGACAGAGTTG-3′. Subsequent hybridization with a probe to the hygromycin sequence confirmed the predicted size of the targeted fragment and that only a single integration had occurred.

The targeted ES cell clones were microinjected into 3.5 day C57BL/6 blastocysts to generate chimaeras. These mice were mated with C57BL/6 mice and transmitted the ES cell genotype through the germline. Screening of heterozygotes produced from multiple ES clones identified animals carrying mutations of the IL-13, IL-4, and IL-5 genes carried on the same chromosome. Mice homozygous for the disrupted alleles were obtained by interbreeding the relevant heterozygotes. IL-13-deficient mice were as described (McKenzie et al., 1998a), IL-9-deficient mice were as described (Townsend et al., 2000), and IL-4/13-deficient mice were as described (McKenzie et al., 1999). The gene-targeted and wild-type animals used in the experiments reported below were maintained on a 129 × C57BL/6 background in a specific pathogen-free environment, with the exception of IL-4KO mice, which were maintained on a C57BL/6 background (Kuhn et al., 1991).

Helminth Infection

Individual mice were inoculated subcutaneously with 500 viable third-stage *N. brasiliensis* larvae and analyzed as described previously (Townsend et al., 2000).

Th2 Pulmonary Granuloma Model

Primary and secondary synchronous pulmonary granulomas were induced by intravenous injection of mice with *S. mansoni* eggs (Townsend et al., 2000).

ELISAs

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 were performed according to the manufacturer's protocol.

Statistical Analysis

Statistical differences between groups were determined using Student's t test. $p < 0.05$ were considered significant.

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