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Signaling via Interleukin-4 Receptor α Chain Is Required for Successful Vaccination against Schistosomiasis in BALB/c Mice

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Although protective immunity in C57BL/6 mice induced by a single dose of the radiation-attenuated schistosome vaccine is believed to be mediated by Th1-type immune responses, we here report that in BALB/c mice protection can also depend upon signaling via the interleukin-4 (IL-4) receptor which conventionally governs the development of Th2-type immune responses. We show that in BALB/c mice deficient for the IL-4 receptor α chain (IL-4Rα$^{-/-}$), which are unresponsive to IL-4 and IL-13, vaccine-induced protection is abrogated compared with that in wild-type (WT) mice. In vaccinated IL-4Rα$^{-/-}$ mice, IL-12p40 production by cells from the skin exposure site was elevated, although gamma interferon (IFN-γ) production in draining lymphoid tissues was similar in WT and IL-4Rα$^{-/-}$ mice. Nevertheless, the effector response in IL-4Rα$^{-/-}$ mice was Th1 biased with elevated IFN-γ in the lungs and higher immunoglobulin G2a (IgG2a) and IgG2b titers but negligible quantities of Th2-associated IgG1 and IgE. Interestingly, levels of IL-4 were equivalent in WT and IL-4Rα$^{-/-}$ mice, indicating that Th2 responses were not dependent upon signaling by IL-4 or IL-13. No differences in the phenotype and composition of the pulmonary effector mechanism that might explain the failure to induce protection in IL-4Rα$^{-/-}$ mice were detected. However, passive transfer of partial protection to naive IL-4Rα$^{-/-}$ mice, using serum from vaccinated WT mice, indicates that Th2-associated antibodies such as IgG1 have a role in parasite elimination in BALB/c strain mice and that signaling via IL-4R can be an important factor in the generation of protection.

The balance of Th1- and Th2-type lymphocyte populations in the host after exposure to infectious agents is crucial to the development of protective immunity or immunopathology. In turn, the differentiation of these polarized lymphocyte populations depends to a great extent upon the relative abundance of various cytokines (e.g., interleukin-12 [IL-12] and IL-4) during the priming of the antigen-specific lymphocyte population by antigen-presenting cells (reviewed in references 52 and 56). While IL-12 and IL-4 are key promoters of Th1 and Th2 cell populations, respectively, they are also mutually antagonistic, with IL-4 capable of inhibiting the expression of the β2 subunit of the IL-12 receptor (62) and IL-12 being responsible for the suppression of IL-4 production in a gamma interferon (IFN-γ)-dependent manner (42).

In the context of protective immunity, we recently demonstrated that the high level of Th1-mediated protection (60 to 70%) induced in C57BL/6 mice by the radiation-attenuated (RA) vaccine model of murine schistosomiasis is dependent upon the presence of endogenous IL-12 (1, 46). Moreover, administration of exogenous recombinant IL-12 during the first few days after vaccination leads to elevated levels of protection, concurrent with increased levels of Th1-associated humoral and cell-mediated immune responses (1, 65, 66). Nevertheless, even in the absence of Th1-type responses (i.e., in vaccinated IL-12p40$^{-/-}$ mice), a reduction in worm burdens of between 35 and 45% was observed, suggesting that Th2-type responses may also have a role in protection in this model (1, 3). Since IL-4 is a major factor in the differentiation of Th2-type cells (24) and, like IL-12, is produced by different cell types of the innate immune response, it is possible that this cytokine contributes to the induction of protective immunity in the RA vaccine model.

Previous studies of the role of IL-4 showed that protective immunity to *Schistosoma mansoni* was not affected by the in vivo administration of anti-IL-4 monoclonal antibody (MAb) 2 to 3 weeks postvaccination and throughout the period of challenge infection, despite a significant reduction in the levels of IL-5 and immunoglobulin E (IgE) (57). However, this study did not address the question of whether IL-4 was important during the induction process in the first 2 weeks after vaccination. Nevertheless, there was also no significant reduction in the levels of protection induced in IL-4$^{-/-}$ mice following exposure to three doses of irradiated cercariae (29), demonstrating that IL-4 was not an important component of immunity to schistosomes. This was verified recently by Hoffmann et al. (23), who showed that protection in IL-4$^{-/-}$ mice exposed to one dose of irradiated cercariae was only slightly reduced compared to that in wild-type (WT) controls. However, doubts have been raised about the interpretation of data obtained using IL-4$^{-/-}$ mice in several models of immunity where the disease outcome was paradoxically unaffected by the absence of IL-4 (31, 37, 49, 53), suggesting that another cytokine may be involved. In this context, IL-13 has been shown to have many overlapping functions with IL-4 (10, 67), including the differentiation of Th2 cells (5, 36), and may thus be responsible for the establishment of Th2-type responses in the absence of IL-4. The similarity in the biological function of IL-4 and IL-13

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is underscored by the finding that IL-13 utilizes the α chain of the IL-4 receptor (IL-4Rα) for signaling (22, 48). Therefore, studies of the role of IL-4 in Th cell differentiation must take account of the possible involvement of IL-13.

Another important concern when analyzing immune responses in intact and gene-disrupted mice is the background strain. Indeed, in a recent study, Bancroft et al. showed that while female IL-4$^{-/-}$ mice on a C57BL/6 background became susceptible to *Trichuris muris* infection compared to their resistant WT cohorts, IL-4$^{-/-}$ mice on a BALB/c background remained resistant (6). In studies using *Leishmania major*, BALB/c mice are more prone to develop Th2-type responses than are C57BL/6 mice (54, 55), and such differences seem to be related to differential regulation of the IL-12 receptor between mouse strains (19, 21, 58, 62). In the context of the RA schistosome vaccine, C57BL/6 mice have been used in nearly all studies (1–3, 12, 23, 26, 29, 40, 41, 43–45, 57, 59–61, 65, 66). However, BALB/c mice can be protected against challenge (reference 14 and this study), although not to the same extent as C57BL/6 mice, and thus may be regarded as moderate and high responders, respectively (25).

In order to reexamine the role of IL-4 (in light of the shared IL-4–IL-13 signaling pathway) in the induction of protective immunity to the RA schistosome vaccine, we have analyzed various parameters of the immune response following vaccination of BALB/c mice with a genetic mutation in their IL-4Rα gene (IL-4Rα$^{-/-}$) (39). Although such mice can produce both IL-4 and IL-13, they do not have a functioning receptor and cannot respond to these cytokines. Our data show that, in the absence of signaling via IL-4Rα, there is a general shift in favor of Th1-type cell-mediated and humoral immune responses, as judged by elevated levels of IL-12, IFN-γ, and IgG2a plus IgG2b antibodies compared to those in WT BALB/c mice. However, contrary to our expectation, levels of protective immunity in vaccinated IL-4Rα$^{-/-}$ mice were dramatically reduced. This demonstrates that signaling via the IL-4R, by IL-4 and/or IL-13, is essential for the development of high levels of immunity. The lack of a Th2-type antibody response in these mice (i.e., no IgG1 or IgE) could be taken as evidence that a significant component of protective immunity induced by a single dose of the RA vaccine in the BALB/c strain is antibody mediated, and this is supported by the observation that serum from vaccinated WT mice can partially restore protection in IL-4Rα$^{-/-}$ mice.

MATERIALS AND METHODS

Host and experimental protocol. Female mice (8 to 12 weeks), with a targeted deletion of exons 7 to 9 of the IL-4Rα chain on a BALB/c background (39), were obtained from the Max Planck Institute for Immunobiology (Frieburg, Germany). These IL-4Rα$^{-/-}$ mice were subsequently bred and maintained in isolators at the University of York animal facility, alongside WT BALB/c and C57BL/6 mice. Groups of mice were vaccinated with optimally attenuated (20-krad gamma irradiation) cercariae of *S. mansoni* via the shaved abdomen (41). In order to determine the level of protection induced, vaccinated (VC) and control (CC) mice were challenged with 200 normal cercariae via the tail 5 weeks after exposure to attenuated larvae. Percent protection was calculated from the mean worm burdens of CC and VC groups 5 weeks after challenge, using the formula (CC − VC)/CC × 100.

In some experiments where study of the dermal immune response was the objective, mice were exposed to irradiated cercariae via the pinnae. Briefly, pinnae of anesthetized mice were immersed in 1.5 ml of cercarial suspension contained in small rectangular glass cells (18 by 18 by 3 mm [height by depth by width, respectively]). The number of nonpenetrant cercariae (25 to 35%) is greater than that after exposure over the abdomen (5%), but the resultant levels of protection are very similar.

Data comparisons were tested for significance by using Student's *t* test. Arithmetic means are shown ± standard errors (SEM). All experiments were repeated between two and four times.

Dermal immune responses. In order to examine immune responses at the skin site of vaccination, two or three mice (providing four to six pinnae) were sacrificed on days 2, 4, and 14 after exposure, and the extent of inflammation was determined by measurement of the pinna thickness using a dial gauge micrometer (Mitutoyo). The pinnae were then cultured in vitro using a technique adapted from that originally described by Milon and coworkers (8). Briefly, pinnae were removed, sterilized in 70% ethanol for 5 min, and then left to air dry. The two faces were separated using forceps and floated on 0.5 ml of RPMI medium containing 10% fetal calf serum, 2 mM L-glutamine, 200 U of penicillin per ml, and 100 μg of streptomycin per ml (RPMI/10) in 24-well hydrophobic culture plates (Greiner Labortechnik, Frickenhausen, Germany), with one face per well and the inner surface in contact with the culture medium. Pinnae were cultured in the absence of any added parasite antigen for 18 h at 37°C with 5% CO_2. The numbers of cells detached from the dermis and free in the culture medium were enumerated (Coulter Z1; Coulter, Luton, United Kingdom). The culture supernatant was collected and stored at −20°C for subsequent cytokine analysis. In all experiments, tissues from naive WT and IL-4Rα$^{-/-}$ mice were similarly prepared to establish baseline levels for dermal immune responses.

Lymphocyte assays. In order to measure lymphocyte activity during priming, axillary lymph nodes (LN) were removed from mice on days 5 and 15 postvaccination when the response is at peak levels (41). The LN cells (2×10^5 cells/well) were cultured in the presence or absence of schistosome antigen from lung-stage larvae (soluble lung-stage antigen preparation [SLAP]) (45) at 50 μg/ml or concanavalin A at 1 μg/ml. CD4$^+$ lymphocytes were isolated from pooled LN cells following labeling with anti-CD4$^+$ magnetic beads and separation on VS+ MACS columns (Miltenyi Biotech, Hamburg, Germany). Eluted purified CD4$^+$ lymphocytes were cultured at 10^5 cells/well together with 2×10^5 naive splenocytes irradiated with 3,000 rads as feeder cells. At 72 h, supernatants were removed from the LN and CD4$^+$ cell cultures for detection of various cytokines, and lymphocyte proliferation was measured by the uptake of [^{3}H]thymidine (18.5 kBq/well; ICN Biomedicals, Thame, Oxon, United Kingdom) into cellular DNA following a further 18-h in vitro culture.

Cytokine ELISAs. Cytokine-specific double-antibody enzyme-linked immunosorbent assays (ELISAs) were used to measure the amounts of IL-4, IL-5, and IL-10 present in the culture supernatants (40, 41, 60). ELISAs were also performed to measure IFN-γ using the R4-6A2 MAb paired with biotinylated XMG1.2 MAb and IL-12p40 using the C15.6 MAb paired with the biotinylated C17.8 MAb (all from Pharmingen). Recombinant IFN-γ obtained from the 211A CHO cell line (60) and recombinant IL-12 (gift of S. Wolf, Genetics Institute, Cambridge, Mass.) were used as internal standards. IL-13 was measured using a specific Quantikine kit (R & D Systems, Abingdon, United Kingdom). The lower limits to detection were 25 (IFN-γ), 20 (IL-12p40), and 10 (IL-4, IL-5, IL-10, and IL-13) pg/ml.

Analysis of pulmonary immune responses. On day 14 postchallenge, at the peak of the pulmonary immune response, leukocytes were recovered from the airways by bronchoalveolar lavage (BAL) as described previously (1, 3). The proportions of the major leukocyte classes within each BAL sample were determined, based on relative size and granularity, using a Coulter XL flow cytometer. BAL cells (2×10^5 cells/well) were cultured in 96-well plates in the presence and absence of SLAP, and supernatants were removed at 48 and 72 h for cytokine measurement. Lobes of lung tissue were taken from individual mice after lavage and fixed in 4% formal saline. Tissue was paraffin embedded, serially sectioned at 7 μm, and stained with Mayer's hemalum-eosin (BDH Laboratory Supplies, Poole, United Kingdom).

Antibody analysis. Serum samples were collected from vaccinated IL-4Rα$^{-/-}$ and WT mice just prior to challenge infection at 5 weeks and before perfusion at 10 weeks. The relative levels of SLAP-specific antibody isotypes in the sera were determined by ELISA over a series of doubling dilutions using horseradish peroxidase-conjugated antibodies (IgG, IgG1, IgG2a, and IgG2b; Zymed Labs, Inc., South San Francisco, Calif.) as described in detail previously (44). Total serum IgE was determined by capture ELISA, and binding levels were compared relative to a standard curve of recombinant IgE (40).

Passive transfer of antisera. Pooled sera from groups of 20 to 30 WT and IL-4Rα$^{-/-}$ mice were obtained 5 weeks after vaccination with 500 irradiated cercariae and frozen prior to use. On day 0, 150 μl of serum from either vaccinated WT or IL-4Rα$^{-/-}$ donor mice was administered intravenously (tail vein) to two separate groups of naive IL-4Rα$^{-/-}$ mice ($n = 5$). Four hours later,

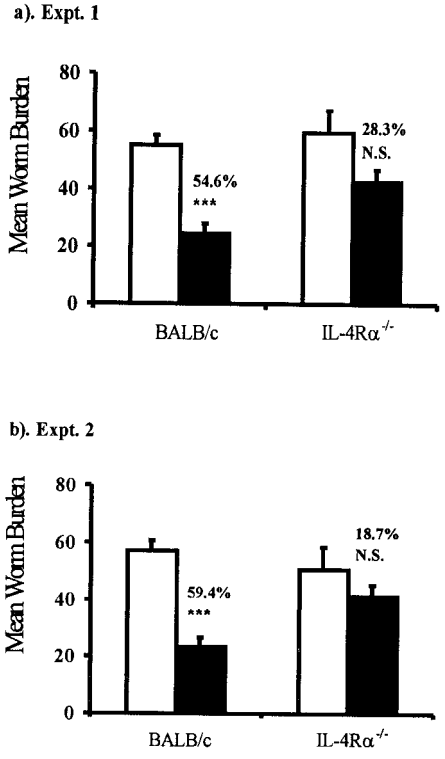

FIG. 1. Worm burdens of challenge control (open bars) and vaccinated and challenged (black bars) mice 5 weeks after challenge infection with 200 normal cercariae in two different experiments. Bars are the means of individual mice (n = 5 to 7) + SEM in each group. Significance values represent differences in worm burdens between vaccinated mice and the relevant control group (***, $P < 0.001$; N.S., $P > 0.05$).

these recipient mice were exposed to 200 normal cercariae via the abdominal skin. On days 3, 7, and 10, repeat doses of sera (150 μl) were administered to the two recipient groups, via the same route. On day 35, the level of resistance was calculated from the mean challenge worm burdens of mice receiving WT serum, compared to those in mice receiving IL-4Rα$^{-/-}$ serum (see above for calculation). IL-4Rα$^{-/-}$ mice which were exposed to 200 cercariae but received no sera served as a further control group.

RESULTS

High levels of protective immunity are induced in WT but not IL-4Rα$^{-/-}$ mice. In order to assess the effect of the IL-4R deficiency on the ability of the host to make a protective immune response, groups of naive and vaccinated, WT and IL-4Rα$^{-/-}$, mice were exposed to a challenge of normal larvae 5 weeks after vaccination. The numbers of challenge worms recovered from control WT and IL-4Rα$^{-/-}$ mice were not significantly different from each other ($P > 0.05$) in either of the two experiments, showing that the disruption to the IL-4Rα signaling pathway had no effect on the maturation of a primary worm burden (Fig. 1a and b). However, while vaccinated WT (BALB/c) mice had significantly lower challenge worm burdens (experiment 1, 54.6%, and experiment 2, 59.4%) than their respective challenge control groups ($P < 0.001$), vaccinated IL-4Rα$^{-/-}$ mice had only slightly lower worm burdens (experiment 1, 28.3%, and experiment 2, 18.7%) which were not significantly different from their challenge control cohorts

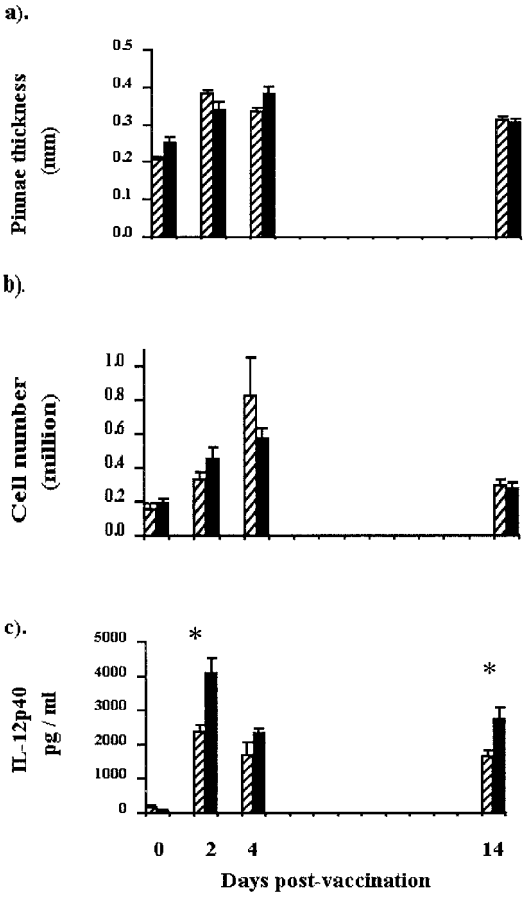

FIG. 2. Dermal immune responses in vaccinated WT (hatched bars) and IL-4Rα$^{-/-}$ (black bars) mice at days 0 (naive), 2, 4, and 14 after vaccination showing pinna thickness (a), recovered cell number (b), and secretion of IL-12p40 (c) by in vitro-cultured pinnae in the absence of added antigen. Bars are the means of results obtained from individual pinnae (n = 6) + SEM.

($P > 0.05$). It appeared, therefore, that the RA vaccine was incapable of inducing significant levels of protective immunity in IL-4Rα$^{-/-}$ mice. A high level of protection was obtained in C57BL/6 mice (63.6%; experiment 1, data not shown) and confirms previous data from our laboratory and others that this strain of mouse is a high responder to the RA vaccine, compared with the BALB/c strain, which is regarded as only a moderate responder (25).

The production of IL-12p40 by dermal cells is elevated in IL-4Rα$^{-/-}$ mice. The skin site of exposure (pinnae) was examined in order to determine any changes, caused by the lack of IL-4R signaling, in the innate immune response following exposure to RA cercariae. A significant inflammatory response was recorded as early as day 2, when the thickness of the pinnae had increased in both IL-4Rα$^{-/-}$ ($P < 0.01$) and WT ($P < 0.001$) mice, compared to groups of naive IL-4Rα$^{-/-}$ and WT animals, respectively (Fig. 2a). The skin remained inflamed for at least the next 12 days, but there was no significant difference in pinna thickness between the two groups at any time point. As a crude measure of the overall cellularity of the pinnae, the number of leukocytes collected in the supernatant after 18 h of in vitro culture was determined (Fig. 2b). There

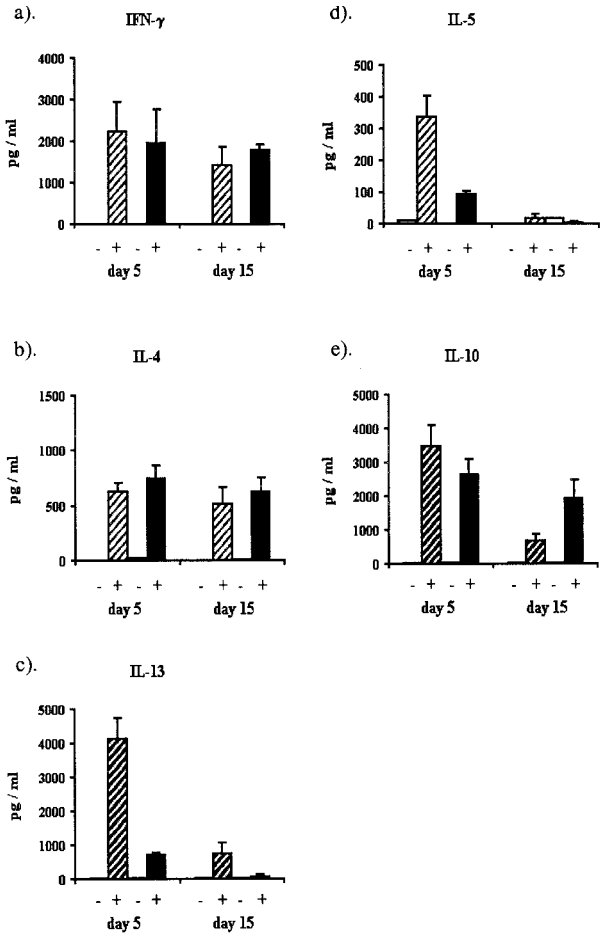

FIG. 3. Secretion of IFN-γ (a), IL-4 (b), IL-13 (c), IL-5 (d), and IL-10 (e) by LN cells obtained on days 5 and 15 postvaccination and stimulated for 72 h with SLAP. Bars are means of individual ($n = 3$ or 4) WT (hatched) and IL-4Rα$^{-/-}$ (black) mice + SEM. Cytokine production was measured by specific ELISAs. Open bars represent cytokine production in the absence of added antigen.

was a rapid increase in the numbers of cells recovered from WT and IL-4Rα$^{-/-}$ mice, and although these had declined by day 14, they were still significantly above naive mouse values ($P < 0.01$). There was, however, no significant difference between the two groups at any time point.

The supernatants obtained following in vitro culture of the split pinnae were assayed for the presence of IL-12p40 as a relative measure of biologically active IL-12p70. A novel finding of our study was that vaccinating parasites are indeed potent stimulators of IL-12 production in the first few days after immunization. An increase in protein secretion was detected by day 2, compared to the day 0 (naive) time point, in both WT and IL-4Rα$^{-/-}$ groups (Fig. 2c). Moreover, secretion of abundant IL-12 was still evident by day 14, reflecting the persistence of RA larvae in the skin (43). Another important observation was that secretion of IL-12p40 by cells in the dermal tissues of IL-4Rα$^{-/-}$ mice was elevated compared to the level from WT mice. The difference was most pronounced on day 2 ($P < 0.05$), when the quantity of IL-12p40 was nearly double the value in WT mice.

Th2- but not Th1-associated cytokine production in the skin-draining LN after vaccination is altered in the absence of IL-4Rα$^{-/-}$. In order to determine the effect on the development of Th lymphocyte subsets in the absence of IL-4 signaling, we measured the cytokine production of cells obtained from the skin-draining LN cultured in vitro with SLAP. At day 5 and day 15, cells from both IL-4Rα$^{-/-}$ and WT mice secreted abundant IFN-γ, the levels of which were not significantly different from each other ($P > 0.01$) (Fig. 3a). Elevated levels of IL-4 were also detected in the two groups of vaccinated mice, and these were very similar at both time points ($P > 0.05$) (Fig. 3b). In sharp contrast, the production of IL-13 was much lower in vaccinated IL-4Rα$^{-/-}$ mice at day 5 and day 15 than in WT mice ($P < 0.05$) (Fig. 3c), although the levels in both groups were lower at the later time point. A similar pattern of secretion was observed for IL-5, where much higher levels were detected in WT than in IL-4Rα$^{-/-}$ mice on day 5 ($P < 0.001$) (Fig. 3d). By day 15, very little IL-5 was detected in either group of mice. Finally, it was observed that, although the levels of IL-10 were lower in IL-4Rα$^{-/-}$ than in WT mice at day 5 (Fig. 3e), the pattern was reversed at day 15, with cells from IL-4Rα$^{-/-}$ mice secreting more IL-10 than their WT counterparts ($P < 0.05$). Experiments using purified CD4$^+$ lymphocytes from pooled LN cells confirmed that virtually all (>95%) of the cytokine production (for all five cytokines tested) was attributable to Th cells (data not shown). Thus, at day 5 postvaccination when the production of IL-5 and IL-13 was most vigorous, there was a decrease in the production of

TABLE 1. Immune responses in the lungs of vaccinated mice 14 days after challenge infection[a]

Type of data	WT	IL-4Rα$^{-/-}$	*p* value
BAL cell population (10^5)			
Total	13.86 ± 1.13	14.58 ± 1.46	NS
Macrophages	9.38 ± 0.71	10.0 ± 0.91	NS
Granulocytes	0.69 ± 0.18	0.46 ± 0.07	NS
Lymphocytes	2.55 ± 0.22	3.97 ± 0.93	NS
In vitro cytokine production (pg/ml)			
IFN-γ	543.7 ± 74.7	1,612.2 ± 65.2	<0.05
IL-4	71.0 ± 7.5	120.2 ± 3.0	<0.01
IL-5	49.5 ± 28.6	0.0 ± 0.0	NS
IL-13	397.0 ± 133.8	523.7 ± 102.4	NS

[a] Cells were recovered from the lungs by BAL 14 days after challenge infection at 5 weeks postvaccination. Data are means for five individual mice in each group ± SEM. Cell types were distinguished on the basis of their size and granularity by flow cytometry. Cytokines were measured by ELISA in supernatants following 72 h of in vitro culture of BAL cells in the presence of SLAP. No cytokines were detected in the absence of added antigen (data not shown). NS, not significant.

these Th2-type cytokines in IL-4Rα$^{-/-}$ mice. Nevertheless, there was no difference in the production of the other archetypal Th2 cytokine IL-4, and there was no corresponding increase in the production of the Th1 cytokine IFN-γ.

Pulmonary effector responses after challenge are not substantially different in IL-4Rα$^{-/-}$ mice. It has been shown previously that protective immunity induced by a single dose of the RA vaccine is associated with a Th1-mediated inflammatory response in the lungs and that this is the main site for challenge parasite elimination in C57BL/6 mice (reviewed in reference 11). Consequently, we examined the immune responses in the pulmonary tissues in vaccinated mice 2 weeks after challenge infection, at the peak of inflammation. The response in the lungs at this time is conventionally associated with a large increase in the number of cells recoverable by BAL, comprising macrophages, granulocytes, and lymphocytes identified on the basis of their size and granularity (60). In the current study, using mice on a BALB/c background, we recovered each of these cell types in numbers similar to those previously reported for intact vaccinated C57BL/6 mice (3), with the major cell population being macrophages (Table 1). Large numbers of infiltrating lymphocytes were also recovered, but there was no statistical difference in the number of any cell type recovered from IL-4Rα$^{-/-}$ compared to that from WT mice.

In order to determine whether the IL-4R deficiency had an effect upon the profile of cytokine production by the BAL cell population, we measured the quantities of IFN-γ, IL-4, IL-5, and IL-13 secreted after stimulation in vitro with SLAP for 72 h (Table 1). The most abundant cytokine was IFN-γ, with significantly higher levels detected in cultures from IL-4Rα$^{-/-}$ than from WT mice ($P < 0.05$), suggesting a shift to a more polarized Th1 response. There was also an increase in the production of IL-4 and IL-13 in the IL-4Rα$^{-/-}$ group, demonstrating that Th2 responses can evolve in the absence of IL-4R-mediated signaling. In contrast, there was no IL-5 in cultures from IL-4Rα$^{-/-}$ mice, although only low levels of IL-5 were detected in some WT mice.

Since we had detected few differences between the immune responses of IL-4Rα$^{-/-}$ and WT mice, we examined the pulmonary inflammatory lesions which form around the challenge larvae, by light microscopy. The number of foci in sections of lungs from the two groups was similar, as were their size and cellular composition (data not shown). In both groups, the cellular infiltrate was largely mononuclear, with few eosinophils or neutrophils in WT mice and almost none in IL-4Rα$^{-/-}$ mice. This contrasts with the Th2-polarized pulmonary response in vaccinated IL-12p40$^{-/-}$ (C57BL/6 strain) (3) and IFN-γR$^{-/-}$ (129 strain) (64) mice, where distinct eosinophilia was detected.

IgG2a and IgG2b production is elevated but IgG1 and IgE levels are reduced in IL-4Rα$^{-/-}$ mice. In addition to measuring parameters of the cell-mediated immune response, we assayed humoral responses in serum samples taken at week 5 (just prior to challenge infection when the immune response should be fully established) and at week 10 (just prior to hepatic perfusion and determination of protection) for differences in the relative levels of antibody isotypes. Anti-SLAP IgG levels were similar in IL-4Rα$^{-/-}$ and WT mice, with the levels in both groups increasing from week 0 (naive) through week 5 to week 10 (Fig. 4). However, IgG isotypes were dramatically different. In WT mice, the levels of antigen-specific IgG2a and IgG2b, both associated with Th1-type immune responses, at weeks 5 and 10 were only marginally higher than at time zero, whereas in IL-4Rα$^{-/-}$ mice the levels of these two isotypes were markedly elevated and significantly higher than in WT mice ($P < 0.05$, week 5; $P < 0.001$, week 10) as expected from the shift to Th1 cytokine production observed for IL-4Rα$^{-/-}$ mice. In contrast, IL-4Rα$^{-/-}$ mice mounted a negligible antigen-specific IgG1 antibody response compared to WT animals, in which the levels of IgG1 were significantly higher ($P < 0.001$) at both time points. IgE was barely detectable in IL-4Rα$^{-/-}$ mice, while in WT mice, over 4,000 and 9,000 pg/ml were present at weeks 5 and 10, respectively. Consequently, IL-4Rα$^{-/-}$ mice had a severe deficiency in their ability to make antibodies associated with Th2-type responses which was compensated for by an increase in the levels of IgG2 subclasses.

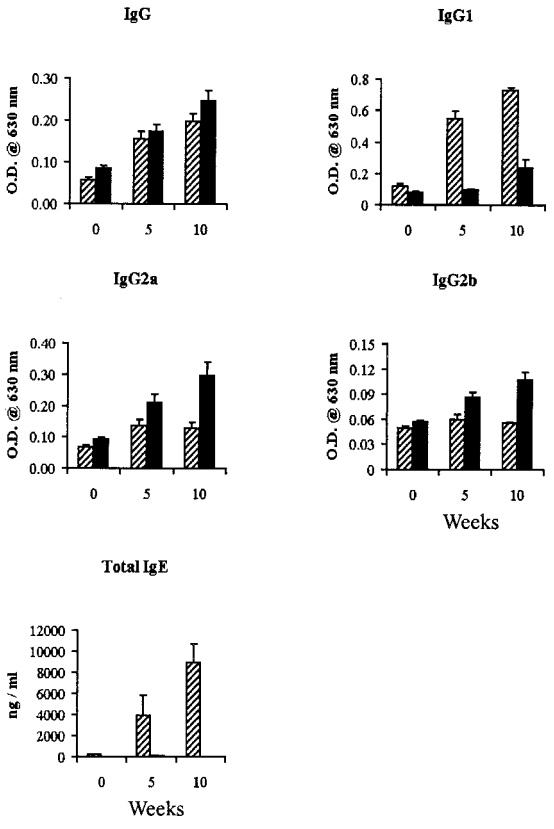

FIG. 4. Antibody production in WT (hatched bars) and IL-4Rα$^{-/-}$ (black bars) mice at week 0 (naive) and at weeks 5 and 10. Levels of SLAP-specific IgG, IgG1, IgG2a, and IgG2b were detected by ELISA, and results are shown at a 1/300 serum dilution. Total serum IgE was calculated from a recombinant standard curve (lower detection limit, 200 pg/ml). Bars are means of individual mice (n = 6 or 7) + SEM. O.D., optical density.

Passive transfer of vaccine sera from WT to IL-4Rα$^{-/-}$ mice restores partial protection against challenge infection. Since the absence of IgG1 and IgE antibodies in the serum of IL-4Rα$^{-/-}$ mice appeared to be associated with the lack of protective immunity, we attempted to investigate whether protec-

TABLE 2. Reduced worm burdens in IL-4Rα$^{-/-}$ mice after passive transfer of serum from vaccinated WT mice[a]

Expt and type of data	Data for group:		
	Group 1 (no serum administration)	Group 2 (administration of IL-4Rα$^{-/-}$ serum)	Group 3 (administration of WT serum)
Expt 1			
Mean worm burden ± SEM	97.7 ± 6.2	106.2 ± 5.5	78.6 ± 2.3
% Reduction compared with group 1		8.7	19.5
P value compared with group 1		>0.05	<0.05
% Reduction compared with group 2			25.9
P value compared with group 2			<0.01
Expt 2			
Mean worm burden ± SEM		73.8 ± 6.1	58.6 ± 3.1
% Reduction compared with group 2			20.5
P value compared with group 2			<0.05

[a] Shown are worm burdens in IL-4Rα$^{-/-}$ mice treated with vaccination serum 5 weeks after challenge with 200 normal cercariae in two different experiments. Serum from IL-4Rα$^{-/-}$ or WT mice exposed to a single dose of irradiated cercariae was delivered by an intravenous route in aliquots of 150 μl on days 0, 3, 7, and 10 after challenge infection. Mean worm burdens are for individual mice ($n = 5$). Significance values represent the differences in the worm burdens between mice receiving serum from WT mice and groups receiving serum from IL-4Rα$^{-/-}$ mice or no serum.

tion could be transferred to IL-4Rα$^{-/-}$ mice using serum obtained from WT (BALB/c) mice 5 weeks after vaccination. Conventionally, immunized mice are exposed to challenge larvae at this time point, and so would expect the appropriate effector responses to be fully established 5 weeks after vaccination. Serum from vaccinated WT and IL-4Rα$^{-/-}$ mice, which had equivalent levels of antigen-specific total IgG antibodies (Fig. 4), was administered to different groups of naive IL-4Rα$^{-/-}$ mice over the period when elimination of migrating larvae is likely to occur. In this respect, IL-4Rα$^{-/-}$ mice receiving vaccine serum from WT mice were protected by 25.9% ($P < 0.01$) and 20.5% ($P < 0.05$) compared with IL-4Rα$^{-/-}$ mice receiving vaccine serum from IL-4Rα$^{-/-}$ mice (Table 2). Moreover, sera from vaccinated IL-4Rα$^{-/-}$ mice conferred no protection since recipient IL-4Rα$^{-/-}$ mice had worm burdens similar to those of IL-4Rα$^{-/-}$ mice which had not received any sera (106.2 ± 5.5 compared with 97.7 ± 6.2; $P > 0.05$).

DISCUSSION

The established dogma that protective immunity to a single dose of the RA schistosome vaccine in C57BL/6 mice is associated with a strong Th1 cell-mediated immune response involving an inflammatory reaction to challenge parasites in the lungs has been supported in many studies using cytokine ablation treatments (57, 59) or cytokine-deficient mice (1, 23, 64). Therefore, we predicted that in the absence of IL-4R signaling, Th1-type responses would increase and that consequently protective immunity would be greater in IL-4Rα$^{-/-}$ than in WT mice. In addition, since our studies were performed using mice on a BALB/c genetic background, which develop only moderate levels of protection compared to the more Th1-polarized C57BL/6 strain mice (25), any shift toward a Th1 phenotype would be expected to lead to greater levels of protection. Instead, we found that IL-4Rα$^{-/-}$ mice developed no significant protection. These results are in striking contrast to those in studies using IL-4$^{-/-}$ mice on a C57BL/6 background, in which high levels of protective immunity were induced (23, 29).

Since our findings were not as predicted, we sought to identify differences in the immune responses shortly after vaccination which might provide an explanation for the abrogation of protection in IL-4Rα$^{-/-}$ mice. The production of IL-12 by accessory cells of the innate response is a key feature for the successful induction of Th1-type immune responses (1, 32, 35), whereas both IL-4 and IL-13 are effective inhibitors of IL-12 production (13, 47, 62). In this context, our data demonstrate that, in the absence of IL-4R signaling, IL-12p40 production was significantly increased in the skin during the first 2 weeks after vaccination when priming of the acquired immune response is passing through a critical phase. It also extends the observations of Noben-Trauth et al. (50), who recorded a twofold-elevated expression of IL-12p40 mRNA in the LN of IL-4Rα$^{-/-}$ mice exposed to *L. major*. Therefore, our data show that IL-4R signaling has an important influence on the cutaneous innate immune response to schistosomes, and it may be envisaged that higher levels of IL-12 in IL-4Rα$^{-/-}$ mice would drive the acquired Th lymphocyte response toward the Th1 phenotype.

In spite of the increased IL-12 production in IL-4Rα$^{-/-}$ mice, the levels of IFN-γ secreted at early time points by LN cells cultured in vitro with schistosome antigen were no higher than in WT mice. A similar failure to observe increased IFN-γ production in the absence of IL-4R signaling has been reported in other models of infection (7, 38, 50, 51). One possible explanation is that the production of IL-12 in WT mice is sufficient to induce a maximal IFN-γ response and that the increased levels in the IL-4Rα$^{-/-}$ mice are superfluous. A more likely explanation is that IL-12R β2 expression is not increased in the IL-4Rα$^{-/-}$ mice on a BALB/c background, which restricts signaling by IL-12. Indeed, Mohrs et al. (38) recently found that IL-4Rα$^{-/-}$ mice exposed to *L. major* had levels of IL-12R β2 mRNA which were indistinguishable from those of their WT BALB/c cohorts but much lower than those of C57BL/6 mice, thus confirming that genetic background determines maintenance of IL-12 signaling (21, 58) and that this is independent of signaling via IL-4 (19).

Nevertheless, at later stages, when the immune effector mechanisms have become established, we observed a nearly threefold increase in IFN-γ production by BAL cells. Although not addressed in this paper, it is possible that other cytokines (e.g., tumor necrosis factor α and IL-1α) rescue the expression

of IL-12R β2 subunit at later times postvaccination and allow a more Th1-biased response to evolve (58). While the increase in IFN-γ was significant, the levels in IL-4Rα$^{-/-}$ mice did not reach those in BAL cell cultures taken from vaccinated and challenged C57BL/6 WT mice, where between 5 and 10 ng/ml is routinely detected at the same time point (e.g., see references 1 and 3). The increased level of IFN-γ in vaccinated IL-4Rα$^{-/-}$ mice is likely to be the major factor favoring the antibody switch to the IgG2a and IgG2b subclasses, also observed in other experimental systems in the absence of IL-4Rα (9, 27, 38, 39, 63). Taken together, our studies show that the lack of effective signaling by IL-4 and/or IL-13 caused a significant shift in the phenotype of the ensuing immune response toward the Th1 phenotype, as judged by cytokine secretion in the lungs and the profile of antibody isotypes.

As a corollary to the increase in Th1 responses, it might be expected that Th2-type responses would be decreased or even abolished in IL-4Rα$^{-/-}$ mice. Indeed, the production of both IL-5 and IL-13 was lower in IL-4Rα$^{-/-}$ mice than in their WT cohorts, and the production of IgE remained below the level of detection (<200 pg/ml) in the former group. We conclude that both IgE and IL-5 are dependent upon IL-4R, although in certain situations IL-5 production can occur in the absence of IL-4Rα (9). Moreover, the absence of significant levels of antigen-specific IgG1 at week 5 is probably due to the failure of IL-4Rα$^{-/-}$ mice to recognize IL-4 rather than IL-13, since IL-13$^{-/-}$ mice mount highly elevated or normal levels of IgG1 (5, 36). Nevertheless, a newly described IL-12-induced switch to IgG1 (18) may result in the low levels of IgG1 detected at week 10 in IL-4Rα$^{-/-}$ mice. Although the majority of Th2-type immune responses were reduced in IL-4Rα$^{-/-}$ mice, the production of IL-4 by the cells in the draining LN was no lower than that in their WT counterparts. This leads us to conclude that a low level of IL-4 production continued in IL-4Rα$^{-/-}$ mice and that, in the absence of its receptor, free IL-4 accumulated in the culture supernatant for subsequent detection by ELISA. This is also probably the cause of the apparent significant increase in the release of IL-4 (and IL-13) by cultured BAL cells from vaccinated IL-4Rα$^{-/-}$ mice.

These results demonstrate that the production of Th2-associated cytokines can occur independently of IL-4 and IL-13, as postulated in earlier studies using *Leishmania* infection (38) and alum as an adjuvant (9), and suggests that alternative factors are responsible for Th2 differentiation. However, it is possible that the residual levels of IL-13 are responsible by signaling via IL-13Rα2 which binds IL-13 independently of the IL-4Rα chain (17), although this has not been identified for IL-4Rα$^{-/-}$ mice so far. Although studies, other than ours, have reported a more substantive effect of IL-4R signaling on the amounts of Th2 cytokines, these have been performed under conditions of extreme Th2 polarization following egg deposition in schistosome-infected mice (27, 28) or infection with a gut nematode, *Nipostrongylus brasiliensis* (7). In contrast, the RA vaccine model in our studies provides a situation with a more limited antigen load which is predisposed to induce a Th1 response and therefore does not amplify the defect in the production of Th2 cytokines.

In seeking to identify the mechanism responsible for the failure to achieve a high level of protection in IL-4Rα$^{-/-}$ mice, we examined the inflammatory foci in the lungs which are thought to be responsible for challenge parasite elimination in vaccinated mice (12). Despite the changes in cytokine production observed for cultures of BAL cells from vaccinated IL-4Rα$^{-/-}$ mice compared to WT mice, we could not see any major differences in pulmonary foci. This contrasts with studies of allergic asthma were dominant Th2-type responses such as eosinophilia, airway hyperresponsiveness, and mucus production were extremely reduced in IL-4Rα$^{-/-}$ mice (20) and studies in schistosome-infected IL-4Rα$^{-/-}$ mice where Th2-type inflammatory granulomas failed to form around eggs in the liver (27). In the present study, it appears that the pulmonary foci in vaccinated IL-4Rα$^{-/-}$ mice remained polarized to the Th1 type but were ineffective at stopping challenge parasites. This is similar to vaccinated TNFRI$^{-/-}$ mice, where the Th1-type pulmonary immune response was not substantially different from that in WT C57BL/6 cohorts but the mice failed to develop protective immunity to the RA vaccine (61). Subtle differences in the complexion of pulmonary foci in vaccinated IL-4Rα$^{-/-}$ mice, which are currently beyond our methods of detection, may explain the failure to eliminate challenge parasites.

The one immunological parameter which did appear to correlate with the failure to induce protection in IL-4Rα$^{-/-}$ mice was the absence of IgG1 and IgE antibodies at the time of challenge. However, since administration of anti-IFN-γ antibodies to vaccinated mice led to greater than 90% abrogation of protection (59), it was previously presumed that there was little scope for a protective role for antibodies after a single vaccination. In addition, studies using gene-disrupted mice exposed to the RA vaccine showed that IgE (30), signaling via the FcR γ chain (26), or antibodies in general (2) were not required for protection. On the other hand, another study proposed a limited role for antibodies by showing that protection was not fully abrogated in vaccinated μMT mice (26). Consequently, we sought to determine a role in protection for antibodies by passively transferring serum from vaccinated WT or IL-4Rα$^{-/-}$ mice to naive IL-4Rα$^{-/-}$ mice and assessing its effect on the maturation of challenge larvae. Since serum from vaccinated WT mice was largely composed of the IgG1 class, while that from the vaccinated IL-4Rα$^{-/-}$ mice contained only IgG2a and IgG2b but little IgG1, we could compare the relative protective capacities of these different isotypes without further purification procedures. The failure to transfer resistance using serum from IL-4Rα$^{-/-}$ mice demonstrates that IgG2a and IgG2b antibody classes are not important effectors of antischistosome immunity. However, the partial reduction in worm burden in mice receiving serum from WT mice exposed to a single dose of the RA vaccine indicates that serum which is rich in IgG1 and IgE does have a role in protection. In this context, previous studies have shown that the protective capacity of serum from multiply vaccinated mice, where protection is mediated by antibodies (2, 23, 26), is associated with the IgG fraction (33) and more specifically the IgG1 subclass (16). Given that very large numbers of mice would be required to provide sufficient donor sera to permit fractionation into the IgG1 and IgE components, we consider it ethically unacceptable to formally prove that the protective capacity of the transferred sera lies with the IgG1 subclass. However, we believe it is unlikely that IgE has a protective role since vaccinated IgE$^{-/-}$ mice remain fully protected (30). In addition, IgE-

dependent responses mediated by mouse eosinophils cannot occur since the low-affinity IgE receptor Fc-εRII is not present on these cells (15).

Our inability to transfer maximal levels of protection (i.e., 55 to 60% observed in WT mice) using serum from singly vaccinated WT mice may arise from administration of insufficient quantities of antibodies, despite serum being given via an intravenous route. Clearly, it is difficult to replicate in IL-4Rα$^{-/-}$ mice the supply of antibodies produced continuously in intact WT mice. Alternatively, we might conclude that protection in WT mice of a BALB/c background, exposed to a single vaccination, is comprised of both antibody- and IFN-γ-mediated effector mechanisms. In this context, the low levels of protection (albeit not significant) seen for IL-4Rα$^{-/-}$ mice after percutaneous vaccination may be due to a residual IFN-γ-mediated component. On the other hand, protection in C57BL/6 mice appears to be more dependent upon IFN-γ (59), while vaccinated IL-4$^{-/-}$ mice on a C57BL/6 background, which also fail to produce IgG1 antibodies, have high levels of IFN-γ and retain their ability to mount high levels of protective immunity (23). Finally, it is possible that IL-13 alone is playing an important role in protection induced by the RA vaccine by a new but, as yet, undefined mechanism as revealed in studies of *Trichuris* (4, 6), or *Leishmania* (34, 39).

In conclusion, the absence of IL-4R signaling has important effects on both the innate and acquired immune responses induced by the RA schistosome vaccine in BALB/c mice, which result in a failure to establish a protective response. The inability to produce sufficient IgG1 antibodies provides the most likely explanation for the lack of protection in IL-4Rα$^{-/-}$ mice. However, until IL-4Rα$^{-/-}$ mice on a C57BL/6 background become available, it is difficult to establish fully the role that host strain plays in controlling the effects observed in this study. Moreover, comparative studies of the efficacy of the RA vaccine in mice deficient for IL-4, IL-13, and IL-4–IL-13 in combination would be instrumental in dissecting the roles of these two important cytokines and may reveal an unappreciated role for IL-13.

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