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IL-4R signaling is required to induce IL-10 for the establishment of T_h2 dominance

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Abstract

The requirement for IL-4 to promote differentiation of naive $CD4^+$ T cells into T_h2 effector cell populations was established by classical *in vitro* studies. More recent *in vivo* data, however, indicate that signaling through the IL-4R is not essential for acquisition of the T_h2 phenotype. In order to reconcile these seemingly contradictory conclusions, we have taken advantage of the ability of the excretory/secretory antigens of the gastrointestinal nematode *Nippostrongylus brasiliensis* to down-regulate T_h1 cell development and drive T_h2 cell expansion. We show that the initial development of IL-4-producing T cells is independent of IL-4R signaling but that the subsequent expansion of IL-4-producing $CD4^+$ T cells in a competitive environment that also contains T_h1 potential is positively influenced by IL-4R signaling. We find that the production of IL-10 is the key IL-4R-dependent factor required to maintain T_h2 dominance and that in the absence of IL-4R signaling, T_h2 expansion can only be achieved by neutralization of T_h1 cytokines. Moreover, in the absence of IL-4R signaling, reduced IL-10 production is due to the lack in expansion of an IL-10^+ T_h2 population, rather than a global defect in the production of IL-10 by $CD4^+$ T cells. Thus, the evolution of T_h2 dominance is achieved at the expense of T_h1 cell development, normally restrained by IL-10 in an IL-4R-dependent manner. We conclude that T_h2 cell development in response to *N. brasiliensis* antigen requires both IL-4 and IL-10 to act in concert on incipient populations of both T_h1 and T_h2 types.

Introduction

IL-4 directs naive $CD4^+$ T cells to differentiate into the T_h2 subset of effector cells (1–4). This subset is characterized by the stereotypic production of a suite of cytokines by committed T_h2 cells, including IL-4, IL-5, IL-10 and IL-13 (5–7). The effects of IL-4 and IL-13 overlap as the receptors for these two cytokines include the common IL-4Rα chain responsible for signal transduction (8). IL-4R signaling, involving the down-stream GATA-3 and STAT6 factors (9–13), is thought to be crucial for the development and maintenance of a T_h2 phenotype. A crucial aspect of the T_h2 subset is its ability to regulate the development of the pro-inflammatory IFNγ-producing T_h1 subset, primarily through the actions of the cytokines IL-4 and IL-10 (14–16).

Although originally classified as a T_h2 cytokine (17, 18), later studies have shown that IL-10 is a key anti-inflammatory regulatory cytokine produced by a wide range of cell types, including both T and non-T cell classes (19, 20). More recently, IL-10 production has been closely associated with an additional type of $CD4^+$ T cell sub-population that has regulatory rather than effector properties (21). Nevertheless, IL-10 remains central to the role of the T_h2 effector population and has proved to be a critical component of resistance and survival during helminth infection (22–24). Thus, both IL-$10^{-/-}$ and IL-4/IL-10-doubly deficient mice exhibit reduced survival during chronic helminth infection (22), while mice deficient in IL-4 alone do not in general show significantly increased mortality (22, 23).

The original paradigm that IL-4 was a prerequisite for induction of the T_h2 subset suggested the necessity of an early non-$CD4^+$ T cell source of IL-4, such as NKT cells (25, 26), eosinophils (27, 28) or mast cells and basophils (28, 29). However, normal T_h2 development has been shown in MHC class I-deficient (NKT deficient) mice infected with *Nippostrongylus brasiliensis* (30) or immunized with *N. brasiliensis* excretory/secretory antigen (NES) from the same parasite (31). Moreover, subsequent studies reported that $CD4^+$ T cells from IL-4Rα$^{-/-}$ animals produce IL-4 during primary *N. brasiliensis* infection (9) or following immunization with

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alum-precipitated model protein antigens (32, 33). Similarly, $CD4^+$ T cells from IL-4R$\alpha^{-/-}$ animals carrying the DO11.10 TCR respond to antigenic stimulation *in vitro* by releasing IL-4 (34). Thus, production of IL-4 can be initiated in the absence of IL-4R signaling.

It is notable that immunization of IL-4R$\alpha^{-/-}$ mice with model antigens in the T_h2-driving adjuvant alum results in strong antigen-specific T_h2 responses as measured by IL-4 and IL-5 (33, 35). However, infection of these mice with the archetypal T_h2-driving gastrointestinal nematode *N. brasiliensis* or the platyhelminth *Schistosoma mansoni* (36) elicits a much more circumscribed level of IL-4 production, an increase in IFNγ production (36, 37) and reduced IL-5, IL-10 and IL-13 (9, 37), compared with congenic wild-type mice. The fact that helminth infections in the absence of IL-4 (23) or IL-4R (36, 37) provoke significant IFNγ responses reveals an underlying potential for T_h1 subset development, possibly due to exposure of the host immune system to microbial mediators (23, 37). This T_h1 capacity is normally tightly restrained in wild-type mice, evidently by mechanisms absent in IL-4R$\alpha^{-/-}$ mice.

Previously, we have reported that mice immunized with NES develop strong T_h2 responses, characterized by increased antigen-specific IL-4, IL-5, IL-10, IgG1, IgE and reduced IFNγ production in a range of genotypes (31, 38, 39). Crucially, NES induction of T_h2 immune responses can overcome the T_h1-driving effects of CFA, due to a specific activity abolished by heat inactivation. T_h2 induction also requires intact MHC class II expression but is independent of B cells (31).

NES effectively reproduces the phenotype of helminth infection, not only with respect to T_h2 polarization but also with respect to the ability to overcome developmental competition from an opposing T_h1 response. We report below our evidence in this system that IL-10 production by $CD4^+$ T cells is dependent on intact IL-4R signaling, and that this in turn is responsible for the maintenance of a dominant T_h2 population in the face of an emerging T_h1-type response.

Methods

Mice

Female BALB/c and IL-4R$\alpha^{-/-}$ BALB/c (40) mice (6–8 weeks old) were bred and maintained at the University of Edinburgh. Female C57BL/6 and IL-10-deficient mice (6–8 weeks old) were obtained from Harlan-OLAC (Oxford, UK). All experiments were performed under the regulations of the Home Office Scientific Procedures Act (1986).

Nippostrongylus and NES

NES antigens were collected from adult worms as previously described (38). Briefly, worms were collected from rats 6 days post-infection and cultured for 7 days in serum-free RPMI-1640 medium containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1% glucose. Supernatants collected between days 1 and 7 of culture were pooled and diafiltrated over a 10-kDa cut off membrane to a concentration of 1 mg ml^{-1}. To produce heat-inactivated *N. brasiliensis* excretory/secretory antigen (hiNES), NES was treated for 15 min at 95°C.

In vitro culture and neutralizing antibodies

Seven days after footpad immunization with 10–100 μg per footpad of NES or hiNES in CFA, popliteal lymph node (PLN) cells were recovered, cultured at 2×10^6 cells ml^{-1} in 200 μl quadruplicate cultures containing either 10 μg ml^{-1} NES or media alone. After 48–72 h in culture, cell supernatants were removed for cytokine assay. For cytokine detection by intracellular staining (ICS), cells were incubated for a further 96 h with 10 ng ml^{-1} recombinant mouse IL-2 (R&D Systems), as has been described (36). Anti-IL-10R mAb for *in vitro* and *in vivo* neutralization used clone 1B1.2 (41). Where stated, cultures with T_h1 neutralized conditions included 10 μg ml^{-1} of antagonistic anti-IL-12 (clone C17.8, BD PharMingen) and anti-IFNγ (clone R4-6A2, BD PharMingen) mAb or control Ig (purified rat IgG, Sigma), whereas IL-10 supplemented cultures contained 10 ng ml^{-1} of recombinant murine IL-10 (BD PharMingen).

Intracellular cytokine staining

Cells from *in vitro* culture or single-cell suspensions were prepared from PLN of immunized mice. Anti-CD4 magnetic microbeads and MACS, separation columns were purchased from Miltenyi Biotec. Cells were stimulated for intracellular cytokine production in the following manner: 2×10^6 cells ml^{-1} were stimulated with 50 ng ml^{-1} phorbol myristate acetate, 1 μg ml^{-1} Ionomycin and 20 μg ml^{-1} Brefeldin A for 6 h. After stimulation, cells were washed in FACS buffer (PBS with 0.5% BSA) and re-suspended at 2×10^6 cells ml^{-1} with 10% normal rat serum for 15 min at 4°C. Cells were then stained for surface markers (anti-CD4-Cy-Chrome, anti-CD25-biotin; BD PharMingen) for 30 min at 4°C, washed 3× in FACS buffer, followed by strepavidin–FITC (BD PharMingen) for 30 min at 4°C, if required. Following a final wash, cells were fixed using a Cytofix/Cytoperm Plus™ kit (BD PharMingen). After fixation, cells were washed in cytoperm/wash solution (BD PharMingen) and stained for 30 min at 4°C with allophycoerythrin (APC)-conjugated anti-IL-4 mAb (11b.11), PE-conjugated anti-IFNγ (XMG1.2) and/or APC- or PE-conjugated anti-IL-10 (JES5-16E3) mAb. All antibodies were supplied by BD PharMingen. In some instances, $CD4^+$ cells were purified before staining using $CD4^+$ magnetic microbeads and MACs separation columns according to manufacturer's protocol (Miltenyi Biotec). After staining, cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and FlowJo software package (Tree Star).

Cytokine assays

Cytokine levels in cell supernatants were determined by ELISA using paired mAbs. Reagents for IL-4 (11B.11 and BVD6-24G2) were a gift of the National Cancer Institute, Frederick, MD, USA; mAbs for IL-10 (JES5-2A5, JES5-16E3) and IFNγ (RA-642; XMG1.2) from BD PharMingen were used as per the manufacturer's instructions.

Statistical analysis

Differences in the mean values of data sets between experimental groups were compared using Student's unpaired *t*-test. *P* values <0.05 were considered to be significant and are indicated by asterisks in the figures.

Results

IL-4 induction by NES but not hiNES

Immunization of animals with NES provokes strong antigen-specific T_h2 recall responses *in vitro*, whereas hiNES treated for 15 min at 95°C shows no such activity (31, 38). Figure 1 demonstrates a significant expansion in intracellular IL-4 expression by $CD4^+$ T cells recovered from draining PLNs at day 5 post-footpad immunization with NES/CFA (A, D), but not in hiNES/CFA (B, D) or naive (C, D) animals. HiNES immunization fails to expand IL-4-expressing cell numbers, although in some mice IFNγ expression rises; in contrast, NES provokes a substantial increase in IL-4^+ T cells, up to 10-fold over background.

Continued expansion of the IL-4^+ population does not occur in IL-4Rα$^{-/-}$ mice

Early IL-4 production was examined in BALB/c and IL-4Rα$^{-/-}$ mice *ex vivo* by ICS of draining lymph node cell populations. No increase in IL-4^+CD4^+ cells was observed in the first 48 h after immunization (data not shown), but by day 3 post-immunization, increased numbers of IL-4^+CD4^+ cells were observed in all mice immunized with NES/CFA (Fig. 2A). Levels increased in BALB/c mice examined at day 7, but failed to do so in IL-4Rα$^{-/-}$ mice. Furthermore, while IFNγ$^+CD4^+$ numbers were consistently lower in NES/CFA compared with hiNES/CFA at all post-immunization time points in BALB/c mice, this was not observed in IL-4Rα$^{-/-}$ mice (Fig. 2A). No consistent differences were observed in the modulation of IL-10^+CD4^+ levels between BALB/c and IL-4Rα$^{-/-}$ mice immunized with NES/CFA.

Both IL-4 and IL-10 antigen-specific recall responses are diminished in IL-4Rα$^{-/-}$ mice

As IL-4R signaling was not essential for initial IL-4 production by the whole $CD4^+$ T cell population, we further determined the requirement for IL-4R signaling in the generation of antigen-specific T_h2 recall responses. As shown in Fig. 2(B), wild-type

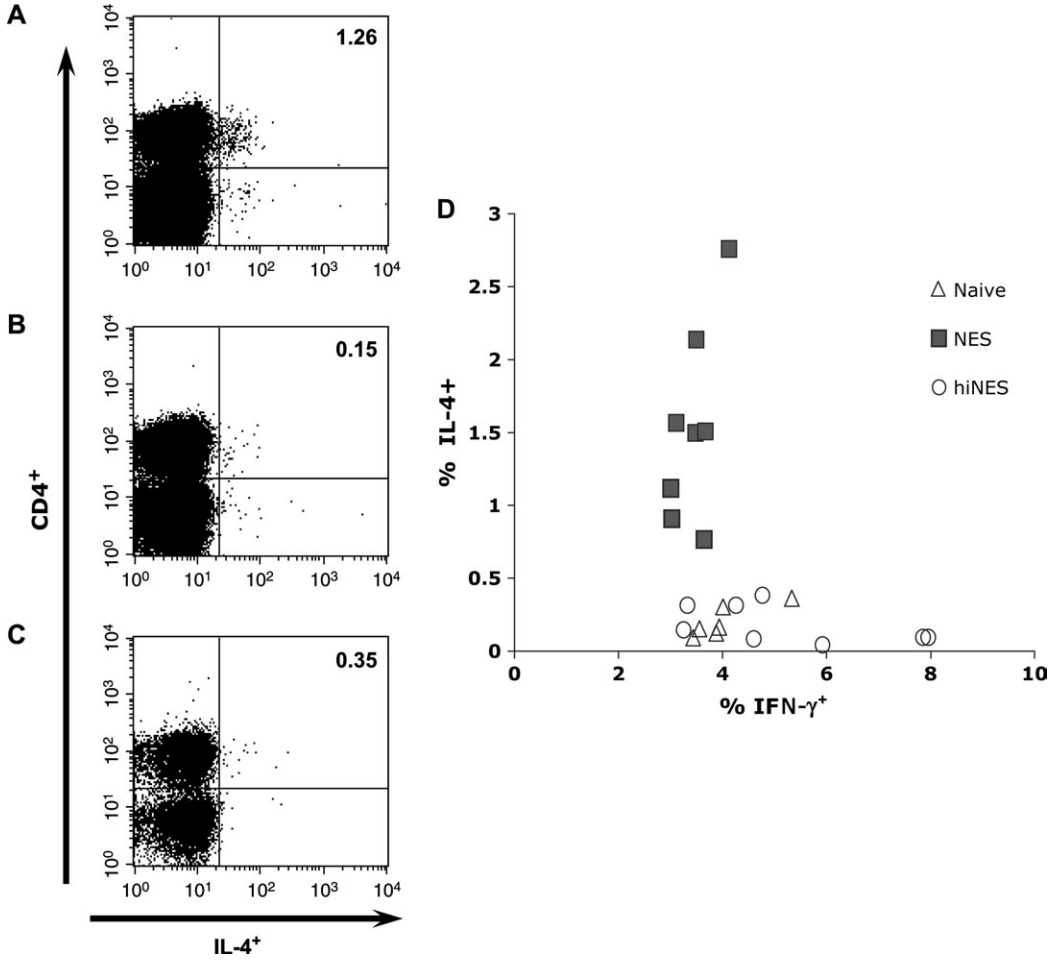


Fig. 1. Early IL-4 responses are induced by immunization with NES, but not hiNES in CFA. (A) Representative intracellular IL-4 staining and FACS analysis of total PLN cells isolated from BALB/c mice 5 days after hind footpad immunization of BALB/c mice with 20 μg of NES in CFA. The percentage of the $CD4^+$ population which stains positive for intracellular IL-4 is shown in the upper right quadrant. (B) As (A) but immunized with hiNES in CFA. (C) As (A) but naive BALB/c mice. (D) Frequency of intracellular IL-4^+ cells within the $CD4^+$ population. Each data point represents an individual mouse, analyzed by ICS as in (A–C), 5 days after immunization with NES (■) or hiNES (○), in comparison to naive PLN cells (△). Increased numbers of IL-4^+CD4^+ T cells and decreased numbers of IFNγ$^+$ $CD4^+$ T cells from the PLN were only detected in NES-immunized mice.

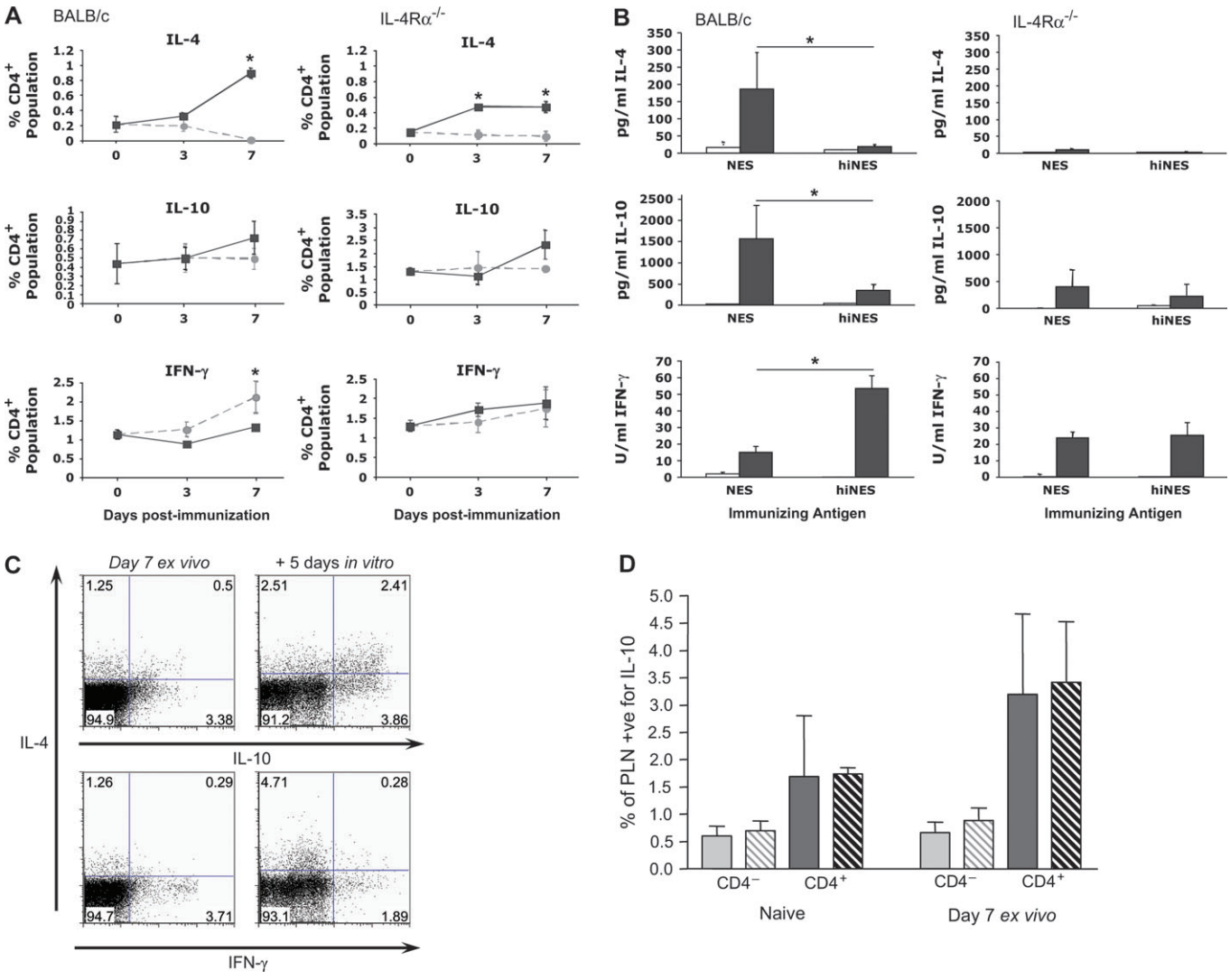


Fig. 2. IL-4R signaling is not required for the initiation of IL-4 production by $CD4^+$ T cells, but is required for expansion of the T_h2 response. (A) PLN cells from BALB/c or IL-4R$\alpha^{-/-}$ mice immunized with 20 μg per footpad of either NES/CFA (■) or hiNES/CFA (●) were recovered at 3 and 7 days after immunization, stimulated for 6 h with 1 μg ml^{-1} ionomycin and 50 ng ml^{-1} phorbol myristate acetate in the presence of 20 μg ml^{-1} Brefeldin A and then stained for surface CD4 and intracellular cytokines. Data are mean ± SD of three individual mice per group. Asterisks indicate significant differences ($P < 0.05$). (B) *In vitro* recall responses of PLN cells isolated 7 days after primary immunization of BALB/c or IL-4R$\alpha^{-/-}$ mice in the hind footpad with 20 μg of NES or hiNES in CFA. PLN cells were challenged *in vitro* with medium alone (□) or 10 μg ml^{-1} NES (■). Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean ± SD of five individual mice per group. Asterisks indicate significant differences ($P < 0.05$). (C) Intracellular cytokine staining profiles of BALB/c PLN $CD4^+$ T cells either stained *ex vivo* (left-hand panels), 7 days after primary immunization with NES/CFA, or PLN cells taken at the same time, after a further 3 days *in vitro* culture with 10 μg ml^{-1} of NES, followed by a further 48 h of culture with 10 ng ml^{-1} IL-2 (right-hand panels). Cells were stained as described in (A), profiles are of individual mice and are representative of three experiments. (D) Frequency of intracellular IL-10 staining in $CD4^-$ and $CD4^+$ PLN cells from BALB/c (solid columns) or IL-4R$\alpha^{-/-}$ (hatched columns) mice. Left, PLN cells from naive animals. Right, PLN cells from mice immunized with NES/CFA and stimulated as described in (A). Data are mean ± SD of three individual mice per group.

in vitro antigen-specific recall responses to NES challenge 7 days post-immunization with NES/CFA compared with hiNES/CFA are characterized by increased IL-4 and IL-10 production and decreased IFNγ as previously reported (31, 38, 39). However, no such differences were observed between immunization groups in IL-4R$\alpha^{-/-}$ mice. Thus, at the level of a differentiated antigen-specific response, NES is unable to induce T_h2 maturation in the IL-4R$\alpha^{-/-}$ genotype.

Extended culture of antigen-stimulated T cells, for a further 96 h in the presence of exogenous IL-2, was then performed to maximize potential cytokine expression (36). Under these conditions, BALB/c PLN cells showed a dramatic expansion in numbers expressing intracellular IL-4, IL-10 or IFNγ measured (Fig. 2C). Bivariate analysis shows that IL-4^+CD4^+ and IFNγ^+CD4^+ T cells are mutually exclusive populations in both *ex vivo* and *in vitro* differentiated populations. However, co-expression of IL-4 and IL-10 while rare in the *ex vivo* population is prominent by day 7 *in vitro*. As IL-10 production from non-$CD4^+$ T cells has been demonstrated (20) and may potentially be independent of IL-4R signaling, we compared intracellular

IL-10 staining in BALB/c and IL-4Rα$^{-/-}$ CD4$^-$ and CD4$^+$ cells (Fig. 2D) 7 days after NES/CFA immunization. We found that the vast majority of IL-10$^+$ cells expressed surface CD4, and that this pattern was maintained in both naive and infected mice in both BALB/c and IL-4Rα$^{-/-}$ genotypes (Fig. 2D). While the overall numbers of CD4$^+$IL-10$^+$ cells were maintained in the IL-4Rα-deficient animals, these cells did not co-express IL-4 *ex vivo* (Fig. 2C).

Although CD4$^+$ cells capable of producing IL-4 were demonstrated *ex vivo* by ICS in early draining lymph node populations from NES/CFA-immunized IL-4Rα$^{-/-}$ mice (Fig. 2A), no significant IL-4 release was detectable even after *in vitro* re-stimulation (Fig. 2B). We hypothesized that a higher dose of NES may be required to suppress the T_h1-driving ability of CFA in the IL-4Rα$^{-/-}$ genotype. Therefore, BALB/c or IL-4Rα$^{-/-}$ mice were immunized with either 10 or 100 μg per footpad of NES and after 7 days antigen-specific recall responses were tested *in vitro*. As shown in Fig. 3(A), while both dose ranges resulted in essentially identical cytokine profiles in BALB/c mice, there was a dramatic difference in the production of IL-4 in the IL-4Rα$^{-/-}$ background, as IL-4 was only detected in the 100-μg per footpad group. Significantly, the higher dose restored IL-4, but not IL-10 production in IL-4Rα$^{-/-}$ mice. Interestingly, while IL-10 release was not restored in the IL-4Rα$^{-/-}$ background, as determined by ELISA (Fig. 3A), IL-10$^+$CD4$^+$ cells were detected by ICS. However, these IL-10$^+$CD4$^+$ cells were either IL-10$^+$IFNγ$^+$ or IL-10$^+$IL-4$^-$, as no IL-10$^+$IL-4$^+$ CD4$^+$ cells could be detected (Fig. 3B).

Neutralization of T_h1 cytokines does not restore a full T_h2 phenotype to IL-4Rα$^{-/-}$ mice

While IL-4R signaling is not an absolute requirement for the production of IL-4, intact IL-4R signaling can increase the likelihood of T_h2 outcome in a mixed T_h1/T_h2 immune response. This may indicate either that IL-4R signaling is required to directly drive expansion of the T_h2 population or that IL-4$^+$CD4$^+$ T cells present in mixed populations fail to

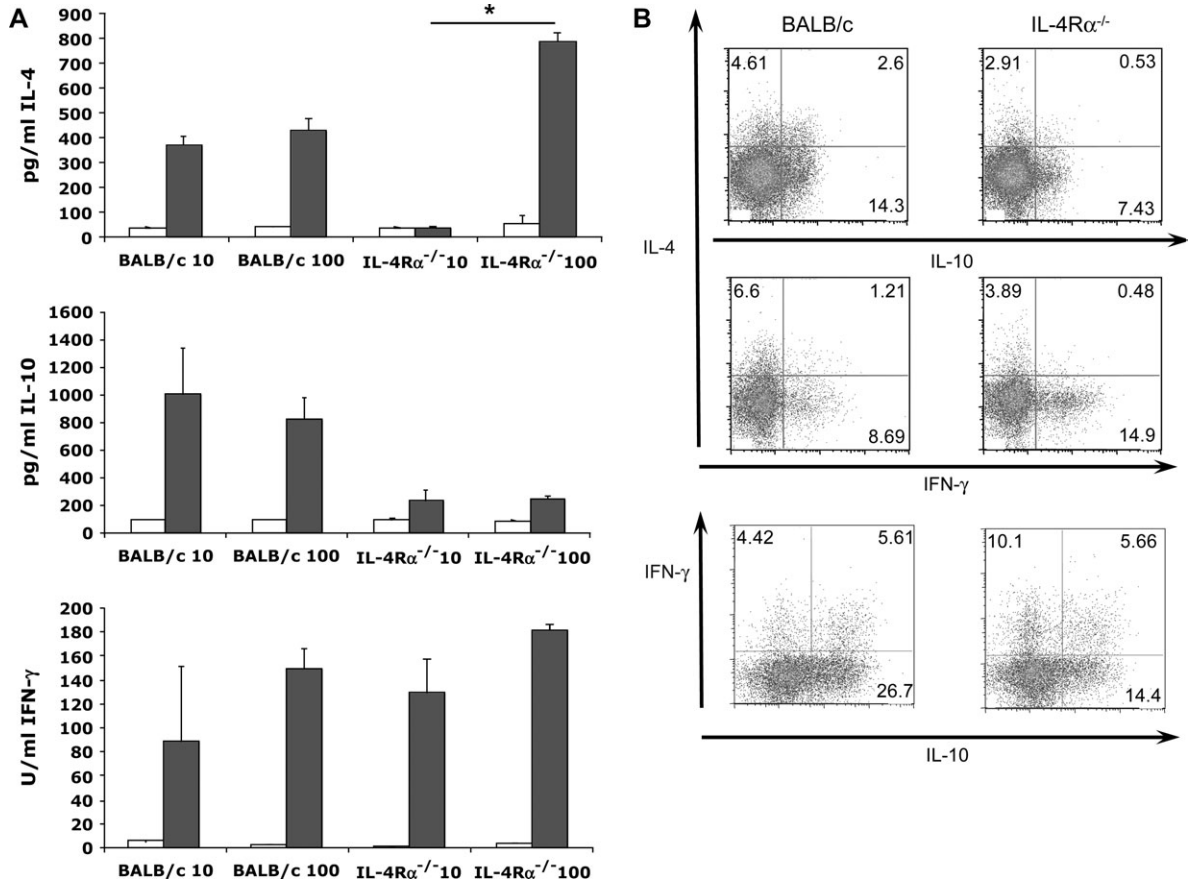


Fig. 3. (A) Increasing the immunization dose of NES results in IL-4 recall responses *in vitro*. *In vitro* cytokine production was assessed in PLN cell cultures from BALB/c or IL-4Rα$^{-/-}$ mice immunized with either 10 or 100 μg per footpad of NES/CFA. PLN cells were stimulated with medium alone (□) or 10 μg ml^{-1} NES (■). Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean ± SD of six individual mice/group. Asterisk indicates a significant difference ($P < 0.05$). (B) IL-10 production in individual CD4$^+$ T cells is not completely abolished in IL-4Rα$^{-/-}$ mice, but IL-4$^+$IL-10$^+$, but not IFNγ$^+$IL-10$^+$ CD4$^+$ cells are absent even after high-dose NES immunization. Intracellular cytokine staining profiles of BALB/c and IL-4Rα$^{-/-}$ PLN CD4$^+$ cells after *in vitro* recall response. Mice were immunized with 100 μg per footpad of NES/CFA, and 7 days after immunization, PLN cells were cultured *in vitro* with 10 μg ml^{-1} of NES for 3 days, followed by a further 48 h of culture with 10 ng ml^{-1} IL-2. Cells were stained as described in Fig. 2(A). Note that when co-staining with anti-IL-4, an APC-conjugated anti-IL-10 was used, while when co-staining with anti-IFNγ, it was possible to use a higher intensity PE-conjugated IL-10.

expand because of inhibition by inhibitory cytokines. To examine if the latter was the case, we measured antigen-specific recall IL-4 responses from primed BALB/c and IL-4R$\alpha^{-/-}$ lymph node cells in culture conditions which neutralized IL-12 and IFNγ. As seen in Fig. 4, under these conditions IFNγ production was completely inhibited, coincident with an increase in IL-4 production in both BALB/c and IL-4R$\alpha^{-/-}$ cell cultures. In contrast, increased IL-10 production after IL-12 and IFNγ neutralization was only observed in cultures of NES-immunized BALB/c cells. This indicated that the removal of elements inhibitory for T_h2 differentiation is sufficient to increase IL-4 production in the absence of IL-4R signaling, but not to restore all characteristics of classical T_h2 cell.

IL-10 deficiency or in vivo treatment with blocking antibody to IL-10R abolishes T_h2 bias

As we found that it was not possible to restore IL-10 production to IL-4R$\alpha^{-/-}$ CD4$^+$ cells, we examined the role of IL-10 in the generation of a T_h2 response by NES. To test if the ability of NES to drive a T_h2 response in the presence of CFA is due to the inherent anti-inflammatory properties of IL-10, we

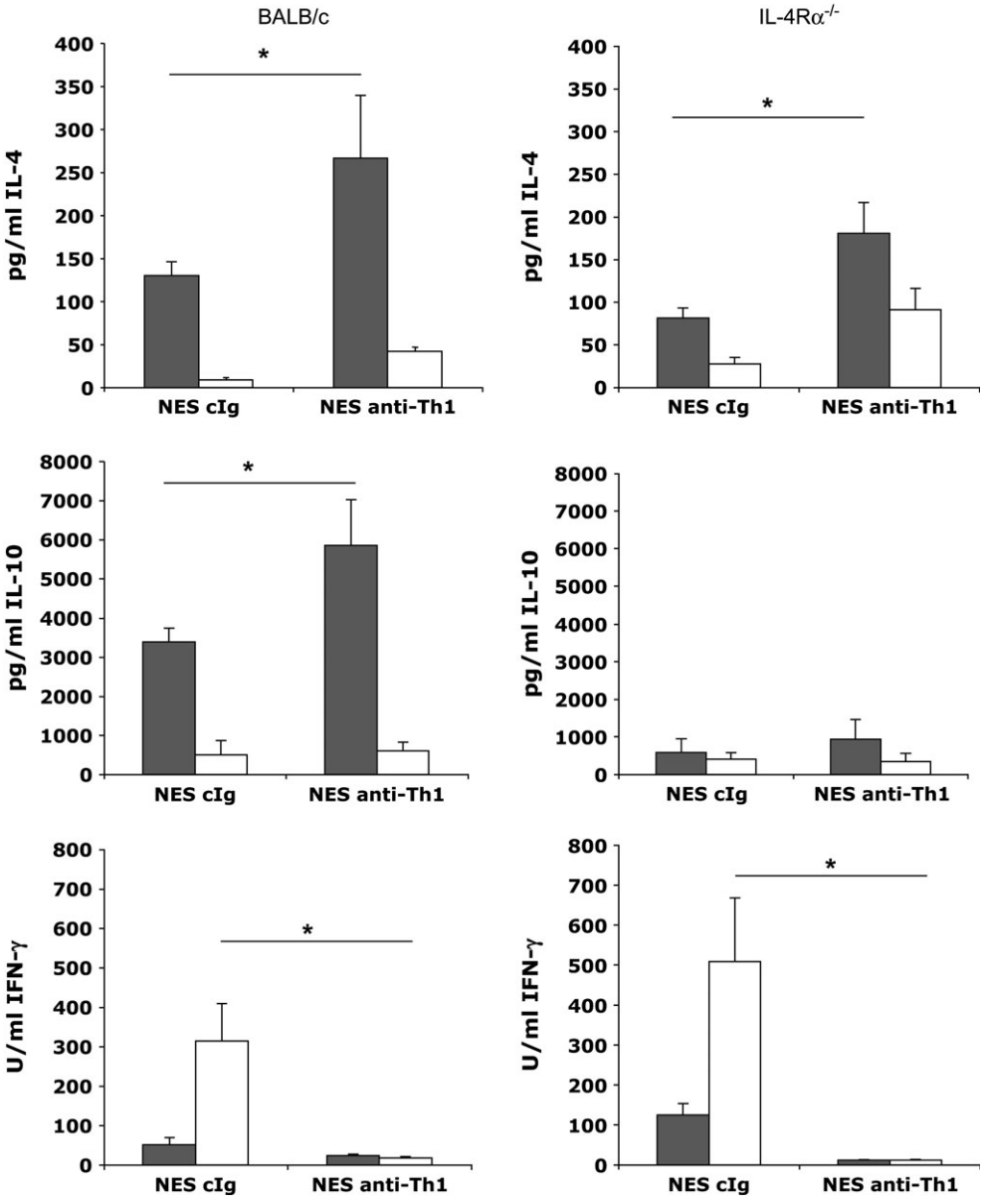


Fig. 4. Neutralization of T_h1-promoting cytokines IL-12 and IFNγ results in increased IL-4 production in both BALB/c and IL-4R$\alpha^{-/-}$ mice after NES immunization, but does not restore IL-10 production to IL-4R$\alpha^{-/-}$ cell cultures. *In vitro* cytokine production was assessed in PLN cell cultures from BALB/c or IL-4R$\alpha^{-/-}$ mice immunized with 100 μg per footpad of either NES/CFA (■) or hiNES/CFA (□). PLN cells were stimulated with 10 μg ml^{-1} of NES for 3 days in the presence of T_h1-neutralizing antibodies (10 μg ml^{-1} of antagonistic anti-IL-12 and anti-IFNγ mAb) or control Ig. Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean ± SD of six individual mice/group. Asterisks indicate significant differences ($P < 0.05$).

examined the response to NES in BALB/c mice in which the IL-10R had been neutralized by mAb treatment. As shown in Fig. 5(A), while mice treated with control antibody responded with antigen-specific IL-4 and IL-10 as expected, mice treated with α-IL-10R showed a dramatic reversion to a T_h1 immune response, characterized by high levels of IFNγ and low levels of IL-4 and IL-10 production. To confirm that this was due to the actions of IL-10, rather than alternate IL-10R ligands, we also immunized IL-10-deficient mice with NES/CFA. In the absence of IL-10, there was reversion to a T_h1 phenotype (Fig. 5B). Thus, the ability to produce or respond to IL-10 at the time of initial antigen exposure is a requirement for the ability of NES to block T_h1 cell development.

IL-10 signaling is required to suppress IFNγ production, but is not an absolute requirement for IL-4 expression

While the experiments above demonstrate that intact IL-10R signaling is required for NES immunization to develop a mature T_h2 response, the data do not distinguish whether continued IL-10R signaling is required to maintain T_h2 responsiveness. To investigate this, we immunized mice with either NES/CFA or hiNES/CFA and examined the resultant antigen-specific recall response in the presence of increased concentrations of blocking anti-IL-10R mAb. As shown in Fig. 5(C), blockade of IL-10R signaling reduces the production of IL-4 by NES-primed T cells and enhances IFNγ expression in both NES- and hiNES-primed groups. However, enhanced IFNγ expression after IL-10R blockade was most dramatic in the hiNES-immunized group. Thus, it would seem that where commitment to the T_h2 subset has been achieved (as in the case of NES), IL-10R signaling is no longer required to maintain the ability to produce IL-4; however, in circumstances where a mixed phenotype T cell memory population is induced (e.g. hiNES), IL-10 plays a role in the suppression of IFNγ production and maintaining the dominance of the T_h2 response. This is confirmed by the observation that IL-10R is not required to maintain IL-4 production when in the case of NES/incomplete Freund's adjuvant (IFA) immunization, where there is minimal competing T_h1 response (Fig. 5D).

Discussion

The pathway by which T cell lineages may become polarized to the T_h2 phenotype has been extensively reported in the literature. The majority of earlier studies concluded that differentiation to a T_h2 profile requires the presence of exogenous IL-4 as well as TCR cross-linking (2, 42–45). Although residual T_h2 responses are found in IL-$4^{-/-}$ mice, these can be attributed to the action of IL-13, which acts on the common IL-4Rα chain to transduce similar signals to IL-4 acting through the STAT6 molecule. T_h2 responses generated independently of IL-4/IL-13 have been demonstrated not only in parasite-infected STAT$6^{-/-}$ mice (13, 36, 46) but also in IL-4R$\alpha^{-/-}$ mice which are unable to respond to either canonical T_h2 cytokine (32, 33, 35). However, the T_h2 responses in these deficient strains of mice is often of a lower magnitude than observed in wild-type mice and is accompanied by an increase in the T_h1 cytokine IFNγ (16, 36, 37, 47). Therefore, while it is clear that IL-4R signaling is not required to generate a T_h2 response in a T_h2-promoting environment, it remains uncertain if a T_h2 response can be successfully mounted in a mixed T_h1/T_h2-promoting environment in the absence of intact IL-4R signaling. In addition, it remains unclear from these studies if T_h2 development requires active innate signals, or if this pathway represents a default in the absence of inhibitory IFNγ and IL-12 p70 (4, 48). The recent finding that the different members of the Notch ligand family direct T_h1 and T_h2 $CD4^+$ T cell lineage fates (49, 50) and that exposure of dendritic cells to helminth-derived products can induce T_h2 responses in the presence of T_h1-promoting stimuli (39, 51) argues against the idea that the generation of T_h2 responses is a simple default process.

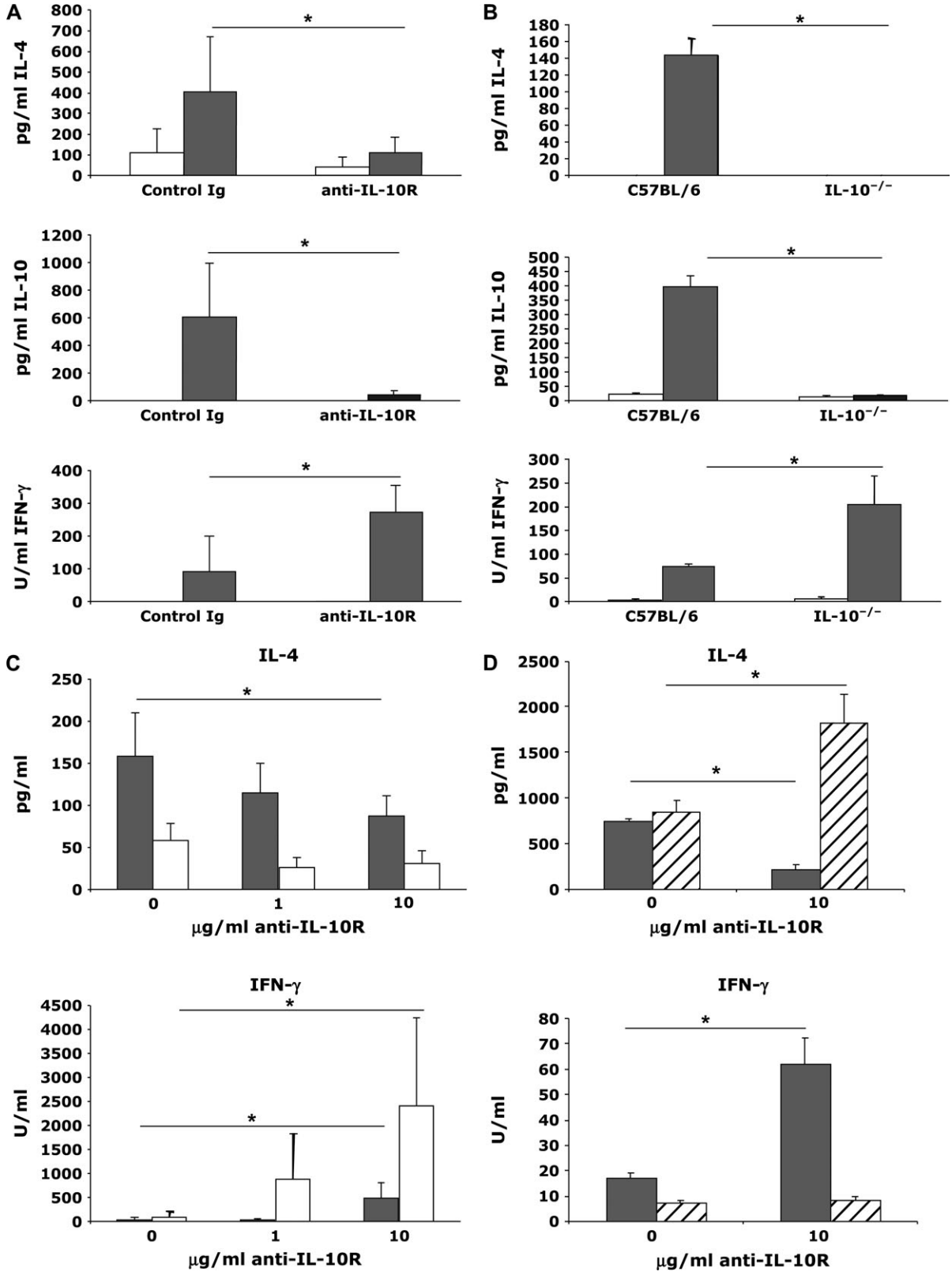
A crucial feature of the naturally T_h2-driving NES antigen in this study is its ability to overcome the T_h1-promoting activity of CFA (31, 38, 39). This allowed us to examine early events in the generation of the T_h2 response in the presence of a competing T_h1-inducing environment. The abolition of IL-4 induction by heat inactivation of NES further confirms that the ability of NES to drive a T_h2 response is an active process. Therefore, as adult stage gastrointestinal nematodes, such as *N. brasiliensis*, reside within a host niche rich in pro-inflammatory microbial products, it is likely that some nematode products have evolved as a means of avoiding undue damage to the host, which could be lethal to both host and parasite (23).

We now present evidence that the ability of NES to stimulate a T_h2 response in the presence of T_h1-promoting CFA is associated with initial IL-4Rα-independent synthesis of IL-4 by $CD4^+$ T cells, but the establishment and maintenance of a dominant, mature T_h2 phenotype requires IL-4Rα-mediated signaling to counteract developing T_h1 response. For example, while IL-4^+CD4^+ cells could readily be detected *ex vivo*, after re-stimulation *in vitro* IL-4R$\alpha^{-/-}$ cells only secrete IL-4 if given an initial high-dose NES immunization. However, IL-4 secretion is readily achieved in IL-4R$\alpha^{-/-}$ mice immunized with alum-precipitated antigens (33, 35). This contrast suggests that in the presence of T_h1 populations generated by CFA immunization, IL-4R$\alpha^{-/-}$ IL-4^+CD4^+ cells are not able to successfully compete. Similarly, the diminished T_h2 response observed in *N. brasiliensis*-infected IL-4R$\alpha^{-/-}$ mice (9, 52) and increased T_h1 response (35) reflects a failure to overcome the T_h1-promoting influences from active infection in the gastrointestinal tract.

Interestingly, while IL-4^+CD4^+ T cells could be detected as early as 3 days post-NES immunization and had expanded by day 7, these cells did not co-express IL-10. Although similar single-positive IL-4^+ or IL-10^+ $CD4^+$ cells have been previously described as 'non-classical' T_h2 cells (53), it is likely the profile of these cells simply reflects their early stage in the progression to a committed T_h2 phenotype. Accordingly, we found co-expression of IL-4 and IL-10 could be induced after antigen-specific re-stimulation *in vitro*. This is in agreement with previous reports in which primary IL-4 production by $CD4^+$ cells is independent of IL-4, whereas the subsequent steps of primary IL-10 production and memory T cell generation both require IL-4 (54–56).

In fact, we observed a general defect in the IL-10 production in the absence of IL-4R signaling, irrespective of T_h1- or T_h2-promoting immune environments. Decreased IL-10 production in the IL-4R$\alpha^{-/-}$ background has also been observed during

Leishmania major infection (57), supporting the idea that our observations are not restricted to gastrointestinal nematode infections. We found reduced numbers of IL-10$^+$CD4$^+$ T cells in IL-4R$\alpha^{-/-}$ mice immunized with NES, and no antigen-specific IL-10 production in primed IL-4R$\alpha^{-/-}$ cell cultures. Even cell culture conditions which effectively neutralized



T_h1-promoting cytokines and thereby increased IL-4 levels failed to restore IL-10 production in IL-4R$\alpha^{-/-}$ cells. This deficit in IL-10 production would appear to be due to the lack of T_h2 IL-4$^+$IL-10$^+$ cells, as IFNγ^+IL-10$^+$ and IL-10 single-positive were readily identified by ICS, although the presence of these IL-10$^+$ populations did not translate to antigen-specific IL-10 production.

The deficiency in IL-10 production in IL-4R$\alpha^{-/-}$ CD4 T cells is likely to be highly significant in functional terms. IL-4 is known to modulate CD4$^+$CD25$^+$ T regulatory cell-mediated suppression of effector cell function (58, 59), contributing to the emerging theme of IL-4 control of regulatory immunological processes. In most immune responses where competing T_h1 and T_h2 populations co-exist within the same microenvironment, the outgrowth of a T_h2 response will clearly require the action of IL-10. Thus, the balance is tipped *in vivo* in favor of pro-T_h1 responses in IL-10-deficient C57BL/6 mice or anti-IL-10R-neutralized BALB/c mice given a primary immunization. In this system as in many others (20, 60), IL-10 acts as a major IFNγ-suppressing cytokine, as shown by IFNγ responses in the presence of IL-10R blockade or IL-10 deficiency. This is in accordance with *in vivo* studies of helminth-infected IL-10-deficient mice, where the major outcome is a dramatic increase in IFNγ production (22, 23, 61–63).

To date, less attention has been paid to the role of IL-10 in T_h2 development. Depending on the model system used, deficiency in IL-10 can result in an increase in representative T_h2 cytokines (22, 62, 64, 65) or a decrease in T_h2 cytokine production (23, 62, 63, 66). We found that when neutralizing IL-10R signaling *in vitro*, the effect on IL-4 production was variable, being dependent on the presence or absence of a competing T_h1 response. For instance, IL-10R neutralization during the antigen-specific recall response resulted in an increase in IL-4 production where the initial immunization was with NES/IFA, while the converse was true following NES/CFA immunization. Thus, while IL-10 can reduce T_h2 recall immune responses, its major effect evident during the primary response is to inhibit the development of T_h1 responses, thereby favoring T_h2 development.

While helminth infections are classically perceived as highly polarizing T_h2 immune responses, increasing evidence suggests an underlying potential for T_h1 outcomes. Certainly, in the absence of IL-10, there is a dramatic increase in IFNγ production, accompanied by increased host death correlating with elevated levels of pro-inflammatory cytokines (22, 23). These reports and the data presented in this paper demonstrate that in competitive T_h1/T_h2 settings, such as immunization with NES/CFA or helminth infections, failure to produce IL-10 favors either a non-polarized response (14, 22) or one dominated by the T_h1 phenotype (23). In the case of helminth infections, this in turn can lead to host death. Paradoxically, as T_h2 immune responses are stereotypically associated with worm expulsion, and compromised T_h2 immune responses permit extended infection in the host, reduction of the IL-10 component of the T_h2 response can lead to host death (23). Thus, the dependence of IL-10 on ligation of the IL-4R has a critical impact throughout the course of infection from initial selection of response phenotype to the final stages of disease outcome.

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Abbreviations

APC	allophycoerythrin
hiNES	heat-inactivated *Nippostrongylus brasiliensis* excretory/secretory antigen
ICS	intracellular staining
IFA	incomplete Freund's adjuvant
NES	*Nippostrongylus brasiliensis* excretory/secretory antigen
PLN	popliteal lymph node

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Fig. 5. Intact IL-10R signaling and IL-10 production are required to initiate T_h2 responses, while at later time points IL-10 controls T_h1 cell development. (A) Contribution of IL-10R function to the initial generation of T_h2 responses assessed by injecting either antagonistic anti-IL-10R or control Ig 24 h before immunization with NES/CFA. *In vitro* recall responses of PLN cells isolated 7 days after primary immunization of BALB/c mice in the hind footpad with 20 μg of NES in CFA. PLN cells were challenged *in vitro* with medium alone (□) or 10 μg ml^{-1} NES (■). Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean ± SD of five individual mice/group. Asterisks indicate significant differences ($P < 0.05$). (B) Effects of IL-10R blockade can be replicated in IL-10-deficient mice. *In vitro* recall responses of PLN cells isolated 7 days after primary immunization of wild-type (C57BL/6) and IL-10$^{-/-}$ mice in the hind footpad with 20 μg of NES in CFA. PLN cells were challenged *in vitro* with medium alone (□) or 10 μg ml^{-1} NES (■). Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean ± SD of five individual mice/group. (C) Effect of blocking IL-10R during T_h1 or T_h2 dominated antigen-specific recall response *in vitro*, assessed by adding increasing amounts of antagonist IL-10R mAb to PLN cell cultures derived from BALB/c mice previously immunized with T_h2-promoting NES/CFA (■) or T_h1-promoting hiNES (□) as described in (A). Asterisks indicate significant differences ($P < 0.05$). (D) Effect of blocking IL-10R during T_h2 recall responses with competing T_h1 response or with a 'pure' T_h2 response. Assessed by adding increasing amounts of antagonist IL-10R mAb to PLN cell cultures derived from BALB/c mice previously immunized with T_h2-promoting NES/CFA (■) or T_h2-promoting NES/IFA (▨) as described in (A). PLN cells were challenged *in vitro* with 10 μg ml^{-1} NES. Cell culture supernatants were removed after 48 h and tested for cytokine production by ELISA. Data are mean + SD of six individual mice/group. Asterisks indicate significant differences ($P < 0.05$).

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