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Cross-regulatory role of interferon-gamma (IFN-γ), IL-4 and IL-10 in schistosome egg granuloma formation: *in vivo* regulation of Th activity and inflammation

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SUMMARY

This study examined the relationship of IL-4, IL-10 and IFN-γ with regard to the local granuloma (GR) and draining lymph node (LN) response to *Schistosoma mansoni* eggs. Synchronized GR were induced in naive and schistosome-infected mice at the vigorous (8 weeks) and late chronic (20 weeks) stages. In LN cultures, IL-10 and IFN production peaked on day 4 and was greatest for 8 week-infected mice. All GR cultures contained IFN, but compared with naive mice IL-10 production was accelerated at 8 weeks and abrogated at 20 weeks, consistent with expansion and abatement of Th2 activity. Cytokine neutralization was performed in egg-challenged, naive mice that were adoptively sensitized with lymphoid cells from 8 week-infected donors. GR size, GR macrophage tumour necrosis factor (TNF) production and egg antigen-elicited IL-2, IL-4, IL-5, IL-10 and IFN were examined on day 4 of GR formation. Anti-IFN augmented GR area by 40%, increased local IL-4 and IL-10, but decreased IFN and TNF production. In corresponding LN cultures, IFN decreased by about 50%, while IL-2, IL-4, IL-10 and IL-5 increased by nearly two-, four-, five- and six-fold, respectively. Anti-IL-10 did not affect GR size or GR cytokines, but increased IFN levels in LN cultures four-fold and decreased IL-2, IL-4, IL-5 and IL-10. Anti-IL-4 abrogated GR area by 40%, along with a reduction in local IL-4 and TNF production. In LN, IL-4 depletion reduced IL-4 and IL-5 by 60–70% and increased IFN levels. These results support the notion of a cross-regulatory network in which IFN inhibits Th2 and IL-10 inhibits Th1 cells. IL-4 fosters Th2 cell differentiation in LN, but also performs a critical recruitment function in the eosinophil-rich schistosome egg-induced GR, whereas IFN contributes to enhanced GR macrophage function.

Keywords granulomas T helper cells *Schistosoma mansoni* IL-4 IL-10 interferon-gamma cross-regulation

INTRODUCTION

The discovery by Mosmann *et al.* that T helper cell subsets could be classified on the basis of patterns of cytokine production has provided for significant advances in the analysis of immune responses [1]. There is mounting evidence that the balance between Th1, IFN-γ, IL-2-producing and Th2, IL-4, IL-5 and IL-10-producing cells can determine the state of resistance to certain infections. For example, resistance to intracellular parasites such as *Mycobacteria* sp. and *Leishmania* sp. depends upon an effective Th1 response, whereas Th2 cells seem to counter the resistant state [2–6]. The balance of Th1 and Th2 cells has been attributed to the phenomenon of cross-regulation [7,8]. Specifically, IFN-γ promotes the differentiation of Th1 cells, whereas products of Th2 cells, IL-4 and IL-10, tend to shift responses to a Th2-like pattern by respectively promoting Th2 cell differentiation and directly inhibiting Th1 cell activity.

Infection with helminth parasites such as *Schistosoma mansoni* often elicits strong Th2 responses [9,10]. The primary pathology of schistosomiasis mansoni is related to the granulomatous hepatitis elicited by parasite eggs. We and others have shown that the hypersensitive granulomatous response to schistosome eggs is largely mediated by Th2 cells, and seems to be regulated by IFN-γ [10–13]. Sher *et al.* provided *in vitro* evidence that T cell-derived IL-10 down-regulated IFN production during

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S. mansoni infection [14]. The present study was undertaken to provide a detailed *in vivo* analysis of the relationship of Th1- and Th2-associated cytokines with regard to local schistosome egg-induced inflammation and regional lymphoid responses.

Using selective *in vivo* depletion of cytokines, we clearly demonstrate that a cross-regulatory network involving IFN and IL-10 is active during the granulomatous response to schistosome eggs, and provide information regarding the differential functions of cytokines in lymphoid tissue and sites of inflammation.

MATERIALS AND METHODS

Animals and infection

Female, CBA/J (The Jackson Laboratories, Bar Harbor, ME) mice were used in all experiments. Mice were maintained under specific pathogen-free conditions and provided with food and water *ad libitum*. Cell donor mice were infected percutaneously with 25–30 cercariae of the Puerto Rican strain of *S. mansoni*. Egg donor mice were infected with 200 cercariae. New Zealand white rabbits were obtained from Hazelton (Kalamazoo, MI) and maintained under specific pathogen-free conditions.

Antigen and egg isolation

Schistosome eggs were isolated aseptically from the livers of heavily infected mice by the method of Coker & von Lichtenberg [15]. A standard preparation of Schistosomal egg antigens (SEA) was obtained from the World Health Organization (Geneva, Switzerland).

Cytokines and antibodies

Recombinant murine IL-10 was obtained commercially (Peprotech, Inc., Rocky Hill, NJ). Recombinant murine IFN-γ was kindly provided by Genentech (San Francisco, CA). Polyclonal anti-murine IL-10 and anti-murine IFN-γ antisera were prepared by monthly immunizations of rabbits with the respective recombinant cytokines emulsified with Freund's complete adjuvant (FCA). Antibody titres and specificity were determined by ELISA. Both anti-IL-10 and anti-IFN had detectable activity to a 1 : 100 000 dilution, and were not reactive with a battery of recombinant murine cytokines which included tumour necrosis factor (TNF), IL-1α/β, IL-2, IL-4, IL-5, MCP-1, and MIP-1α. *In vitro* neutralizing activity of anti-IL-10 was determined by its capacity to block IL-10-mediated suppression of macrophage IL-6 production [16]. Neutralizing activity to anti-IFN was determined by its capacity to block IFN-mediated induction of IA antigen expression.

Neutralizing monoclonal rat anti-murine IFN was prepared from the XMG-6 hybridoma cell line kindly provided by Dr F. Finkleman (NIH, Bethesda, MD). Neutralizing monoclonal rat anti-murine IL-4 was prepared from the 11B11 hybridoma (ATCC, Bethesda, MD).

Adoptive sensitization and depletion protocol

Mice were sensitized by adoptive i.v. transfer of 60 million spleen and mesenteric lymph node cells (2 : 1 mix) in 0·5 ml from syngeneic donor mice at the vigorous (8 week) stage of *S. mansoni* infection. One day later mice were challenged with 3000 schistosome eggs by tail vein injection in 0·5 ml PBS and given an i.p. injection of 1 mg of immunoaffinity-purified MoAbs or control rat IgG in 0·5 ml of PBS. Depletions with polyclonal antisera were performed using 5 mg of protein A purified IgG. Four days after challenge, lungs and lymph nodes were excised and cultures prepared as described below. In some experiments, lungs were fixed in 10% buffered formalin for morphometric studies.

Granuloma and lymph node culture

Groups of mice were killed on 2, 4, 8 and 16 days after egg embolization. Following perfusion with cold RPMI, lungs excluding trachea and major bronchi were excised, placed in cold RPMI medium, then homogenized in a Waring blender with a narrow-bottomed stainless steel cup. Granulomas were collected over a sterile stainless steel mesh (no. 100) and rinsed with cold RPMI. Intact lesions were suspended to 750/ml of RPMI medium containing 10% fetal bovine serum (FBS), 100 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI–FBS), then cultured in the presence or absence of 5 μg/ml SEA at 37°C in a 5% CO_2, humidified atmosphere. Supernates were collected by centrifugation at 24 h and 48 h and frozen at −40°C.

Granuloma macrophages were obtained by digestion of intact granulomas in a membrane-sterilized solution of RPMI–FBS containing 1000 U/ml type IV collagenase (Sigma, St Louis, MO). Following 30 min incubation in a 37°C shaker water bath at 120 cycles/min, the digest was passed through a stainless steel mesh (no. 100) and washed four times in RPMI–FBS. Macrophages were isolated by 2 h adherence and were cultured on 35-mm plastic dishes as above in RPMI–FBS in the presence or absence of 1 μg/ml lipopolysaccharide (LPS) (Sigma, *E. coli* 0111 type). Supernates were collected at 48 h and stored as above. Adherent cells on dishes were stained and the total number of adherent macrophages was counted in order to normalize cytokine production. Adherent cells were >90% macrophages based upon morphology and non-specific esterase staining. To determine cell composition, cytospin preparations of dispersed granuloma (GR) were Wright stained and 200 cell differentials performed.

Mediastinal lymph nodes were collected at the time of lung harvest and teased into single-cell suspension. After washing, the cells were cultured in RPMI–FBS at 5×10^6/ml in the presence or absence of 5 μg/ml SEA, then cultured as above for 36 h. Supernates were collected by centrifugation and stored at −40°C.

Cytokine measurement

IL-2 was measured by the standard CTLL-1 proliferation assay using the method of Tada *et al.* [17] with the addition of anti-IL-4 receptor and anti-IL-4 antibodies (Genzyme, Cambridge, MA) to block interference by IL-4. Recombinant murine IL-2 was used as a standard, and sensitivity was 1 U/ml. IL-4 was measured by a standard assay using the CT.4S IL-4-dependent T cell clone developed by Dr W. E. Paul [18], and sensitivity was 0·5 U/ml. Recombinant murine IL-4 standard was obtained from Genzyme. IL-5 was measured by ELISA using dual MoAbs TRFK-4 and TRFK-5 (ATCC) as previously described [19]. Sensitivity was 250 pg/ml, and recombinant murine IL-5 (Genzyme) served as a standard. IFN-γ was measured by ELISA as described [20] and using a capture antibody derived from the XMG-6 clone, kindly provided by Dr F. Finkelman (NIH); sensitivity was 50 pg/ml. IL-10 and TNF were determined by ELISA using commercially available reagents (Pharmingen, San Diego, CA); sensitivity was 50 pg/ml.

Granuloma measurement

Granuloma area was measured blindly in haematoxylin and eosin-stained sections using a morphometer and software program (The Morphometer; Woodshole Educational Associates, Woodshole, MA). A minimum of 20 lesions were measured per lung.

Statistical analysis

Student's *t*-test was used to compare control with treatment groups. $P > 0{\cdot}05$ was considered to indicate lack of significance.

RESULTS

Dynamics of local and regional IL-10 and IFN production during synchronized schistosome egg granuloma formation

We previously reported that the pattern and levels of cytokines produced by GR and lymph nodes (LN) changes dramatically during the course of chronic murine schistosomiasis [11,21]. As part of the present study, we likewise established the relative participation and time course of IL-10 and IFN production in naive and infected mice. Synchronized pulmonary GR were induced by i.v. challenge with schistosome eggs, then IL-10 and IFN production by GR and draining LN cultures was assessed over a 16-day study period. As shown in Fig. 1, IL-10 and IFN levels peaked at 4 days in LN cultures of naive and 8 and 20 week-infected mice. The 8-week stage cultures produced greater amounts of both IL-10 and IFN than 20 week-infected and naive mice. Compared with LN, GR cultures produced modest levels (note scale) of IL-10 and IFN, but distinctly different patterns were observed. The 8-week GR cultures produced detectable IL-10 predominantly on days 2 and 4, but only trace levels were detected in 20-week GR cultures on day 8. Primary GR formation was associated with increasing IL-10 levels as lesions reached their maximum size at 16 days. Unlike IL-10, levels of IFN in GR cultures seemed comparable among groups. However, the trend of higher IFN levels in primary GR cultures at 2–8 days should be noted, since these lesions are smaller than those of infected mice and this probably reflects an absolute increase in production if GR size and cellularity are considered.

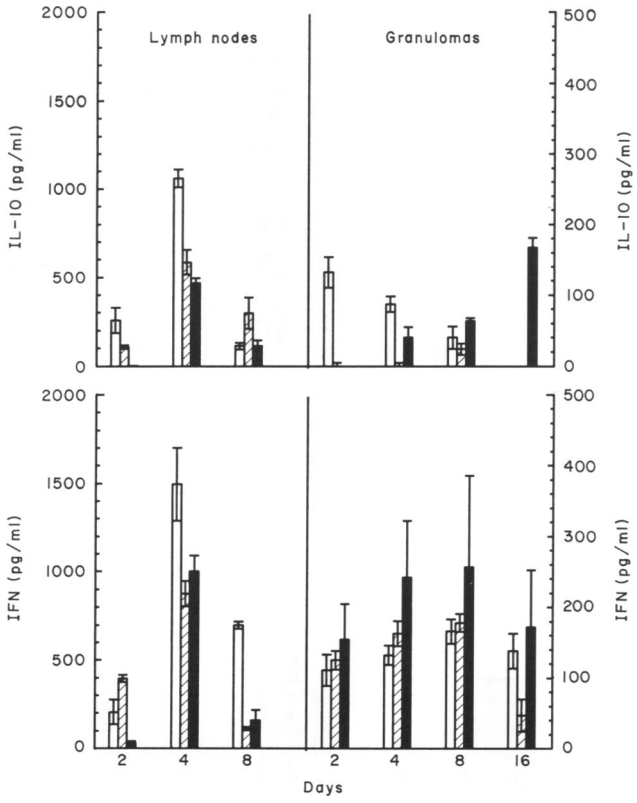

Fig. 1. IL-10 and IFN-γ production by draining lymph nodes (LN) and cultured granulomas (GR). Naive, vigorous (8 week) and modulated stage (20 week) *Schistosoma mansoni*-infected mice were challenged with 3000 eggs intravenously, then draining mediastinal LN and GR were collected at the indicated time points and cultured as described in Materials and Methods. Bars show means ± s.e.m. of egg antigen-elicited cytokine derived from three experiments. Upper panel IL-10; lower panel IFN. Data not shown for 8- and 20-week mice at day 16. □, Eight week; ▨, 20 week; ■, primary.

Effect of cytokine depletions on granuloma size and cytokine production

The above studies indicated that IL-10 and IFN activities were greatest in 8 week-infected mice. We previously demonstrated that the vigorous secondary GR response could be effectively transferred to naive mice with 8 week donor lymphoid cells [21]. Such animals lack the presence of the background granulomatous hepatitis present in infected mice, hence sites of extraneous cytokine production and other potential sources of eggs are eliminated during *in vivo* manipulations. Using this approach, we examined the relative contribution of IL-4, IFN and IL-10 to vigorous stage GR formation in adoptively sensitized mice that had been passively immunized with cytokine-neutralizing antibodies. As shown in Fig. 2, depletion of IL-4 using MoAb abrogated GR area by 40%. Conversely, depletion of IFN with MoAb resulted in a nearly 40% augmentation. Treatment with anti-IL-10 did not significantly affect GR size. Differentials of dispersed granuloma cells indicated that IL-4 depletion primarily reduced eosinophils, whereas IFN depletion did not significantly change the percentage (control, 42 ± 5; anti-IL-4, 15 ± 10; anti-

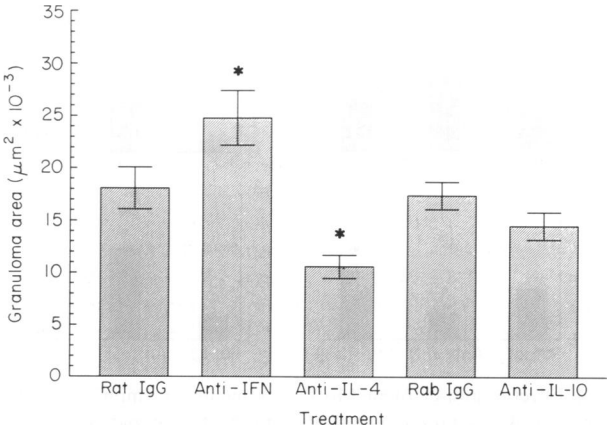

Fig. 2. Effect of anti-cytokine treatments on *Schistosoma mansoni* egg granuloma formation. Granulomas (GR) were induced in passively immunized mice as described in Materials and Methods and examined on day 4. Bars are means ± s.e.m. derived from five mice. A minimum of 20 GR were measured per mouse. $*P < 0{\cdot}05$.

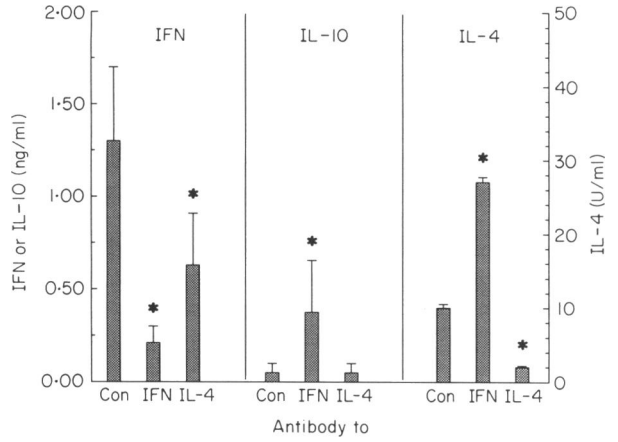

Fig. 3. Effect of anti-cytokine MoAb treatments on intact granuloma (GR)-derived cytokines. GR were induced in passively immunized mice as described in Materials and Methods, then isolated and cultured on day 4. Bars are means ± s.e.m. of egg antigen-elicited cytokine assayed in 24-h supernates. Data are from a representative experiment. In each experiment GR of three to four mice were pooled and cultured. $*P < 0{\cdot}05$.

IFN, 30 ± 7). Anti-IL-10 also tended to decrease eosinophils but did not affect GR size.

Figure 3 shows the levels of cytokines in cultures of GR from control and MoAb-treated mice. Anti-IFN profoundly abrogated local IFN production, and caused a two to three-fold augmentation of IL-10 and IL-4 production that was in part commensurate with the increased GR size. Anti-IL-4 strongly abrogated local IL-4 production, but did not significantly change the already low IL-10 levels. It also decreased IFN levels, possibly as a result of the decreased GR size. IL-2 levels were generally less than 1 U/ml in all GR cultures (data not shown). Treatment with the polyclonal anti-IL-10 did not change local GR cytokine profiles (data not shown).

In order to determine the effect of cytokine depletions on effector cell function, we assessed the capacity of isolated GR macrophages to produce TNF. Anti-IFN treatment decreased

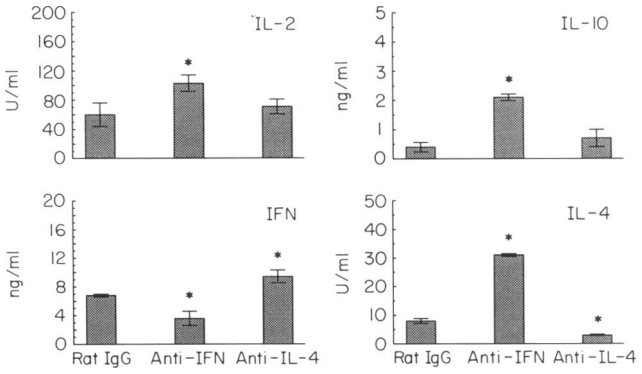

Fig. 4. Effect of anti-cytokine MoAb treatments on lymph node (LN)-derived cytokines. Granuloma (GR) were induced in passively immunized mice as described in Materials and Methods, then mediastinal LN were harvested and examined on day 4. Bars are means ± s.e.m. of egg antigen-elicited cytokine. Data are representative of three separate experiments. In each experiment LN of three to four mice were pooled and cultured. $*P < 0{\cdot}05$.

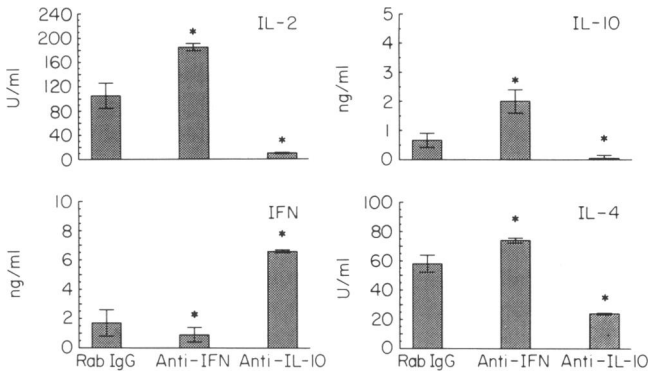

Fig. 5. Effect of polyclonal anti-cytokine antibody treatments on lymph node (LN)-derived cytokines. Granuloma (GR) were induced in passively immunized mice as described in Materials and Methods, then mediastinal LN were harvested and examined on day 4. Bars are means ± s.e.m. of egg antigen-elicited cytokine. Data are representative of three separate experiments. In each experiment LN of three to four mice were pooled and cultured. $*P < 0{\cdot}05$.

macrophage TNF production, whereas anti-IL-10 had no effect (66 ± 12% and 110 ± 9% of control, respectively). Surprisingly, anti-IL-4 treatment also decreased TNF production (70 ± 11% of control).

Effect of cytokine depletions on LN cytokine profiles

The draining mediastinal LN of mice subjected to anti-cytokine antibody treatments were likewise cultured in the presence of egg antigen, and supernates were analysed for IL-2, IFN, IL-10 and IL-4. Figure 4 shows the effects of depletions using the MoAbs. Anti-IFN concomitantly augmented IL-2, IL-4 and IL-10 by nearly two-, four- and five-fold, respectively. In contrast, IFN production decreased by nearly 50%. Anti-IL-4 treatment decreased IL-4 levels by about 60%, and tended to augment IFN-producing cells, but had no significant effects on IL-10 or IL-2 production. Polyclonal anti-IFN had a similar

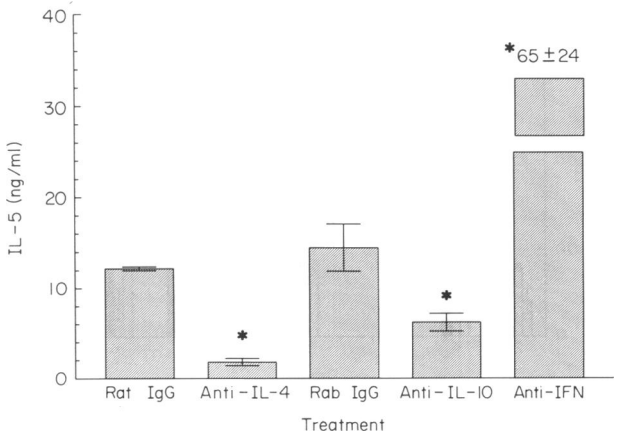

Fig. 6. Effect of anti-cytokine antibody treatments on lymph node (LN)-derived IL-5. Granuloma (GR) were induced in passively immunized mice as described in Materials and Methods, then mediastinal LN were harvested and examined on day 4. Bars are means ± s.e.m. of egg antigen-elicited cytokine. Data are representative of three separate experiments. In each experiment LN of three to four mice were pooled and cultured. $*P < 0{\cdot}05$.

effect to the MoAb, augmenting IL-2, IL-4 and IL-10 (Fig. 5). In contrast to its minimal effect on GR, anti-IL-10 had profound effects in the LN, causing a four-fold increase in IFN production and sharp declines in IL-2, IL-4 and IL-10 production. Figure 6 illustrates that levels of IL-5 were also profoundly affected by these treatments. Anti-IL-4 and anti-IL-10 reduced IL-5 by 60% and 70%, respectively, while anti-IFN enhanced IL-5 production six-fold. The above results provided direct *in vivo* evidence that IFN actively regulates Th2-like cells, and IL-10 regulates IFN-producing cells within LN draining sites of inflammation.

DISCUSSION

There is growing evidence that the antigens of *S. mansoni* eggs induce a strong Th2 response, and that these cells play a critical role in the inflammation associated with tissue deposited eggs [10,11,13,20–23]. Likewise, several studies suggest that Th1 and Th2 cells produce cross-regulatory cytokines that are mutually inhibitory [7,8]. Specifically, IL-10 inhibits Th1 cell expression, whereas IFN and IL-12 inhibit Th2 cells. The present study supports the notion of a cross-regulatory cytokine network, and provides novel information regarding the role of IL-10 in local inflammatory and regional lymphoid responses to *S. mansoni* eggs.

The first part of our study analysed the regional and local expression of IL-10 in mice with primary, secondary and modulated states of immune responsiveness to *S. mansoni* eggs. Our kinetic analysis of LN-derived IL-10 in naive mice was consistent with *de novo* maturation of Th2 cells as proposed by Vella & Pearce [22]. In contrast, vigorous stage LN showed enhanced and accelerated IL-10 expression consistent with expansion of a memory Th2 population [24]. As we previously reported for IL-4, antigen-elicited IL-10 and IFN production was enhanced during the vigorous stage (8 weeks), then down-regulated in the modulated stage of infection ($\geqslant$ 20 weeks) [11]. This finding indicates that both IL-10 and IFN-producing cells are generated during the response to *S. mansoni* eggs, and are subject to immunomodulation in the chronic stage of infection as we have previously proposed [21].

Our depletion studies provide novel information regarding differential local and regional cytokine activities. Specifically, IFN and IL-4 appeared to regulate GR formation as well as LN maturation events, while IL-10 was primarily active in LN. Compared with LN, GR produced low levels of IL-10, usually less than 200 pg/ml. This contrasts with IL-4, which can achieve quite substantial levels in GR cultures, sometimes surpassing those of LN cultures [21]. Taken together with our finding that IL-4 depletion abrogates GR formation better than IL-10 depletion, it suggests that IL-4 has a local proinflammatory function but IL-10 has greater influence within lymphoid tissues and may be selectively down-regulated at sites of inflammation. IL-10 appears to be analogous to IL-2, which similarly displays a weak local and strong regional expression during the response to eggs [11,23]. Anti-IFN treatment augmented GR size and LN-derived Th2-associated cytokines, indicating that IFN had local and regional regulatory activity. It should be noted that the present study employed the XMG-6 monoclonal and high titre polyclonal anti-IFN antibodies, whereas an earlier study using the less potent R46A2 antibody did not show the augmenting effect on GR size [11].

The observed effects of IFN and IL-10 depletion support the role of these mediators as key cross-regulatory cytokines in determining the development of Th1- and Th2-like responses as described in other parasite models [8,25,26]. Based upon our results, IFN inhibits Th2 activity in LN and GR. This is fully consistent with the findings of Gajewski *et al.*, demonstrating that IFN selectively inhibits Th2 cell clonal proliferation *in vitro* [27]. In contrast, IL-10 appeared to inhibit IFN-producing cells and promote Th2 activity. The latter was most probably due to its restraining effect on IFN, since IL-10 does not seem to promote directly Th2 cell differentiation *in vitro* or *in vivo* [28]. IL-4 is thought to be important in driving Th2 differentiation [29,30], and indeed our depletions lend support to this notion, since IL-4/IL-5 production in LN was reduced by anti-IL-4 treatment. Surprisingly, other than a modest augmentation of IFN, IL-4 depletion did not affect IL-2 or IL-10 levels as did IL-10 depletion. Perhaps IL-4 acts mainly on a subpopulation of uncommitted precursors, and the activity of committed Th2 memory cells is not affected by IL-4 depletion. Thus, only a *de novo* generated portion of IL-4/IL-5-producing cells would be abrogated, while Th0 and Th1 cells would be enhanced. In addition, we cannot rule out the possibility that IL-4 selectively induces IL-4/IL-5 gene expression.

The LN cytokine profiles following depletions also provide important *in vivo* information regarding T cell maturation during the secondary response to schistosome eggs. IFN-γ depletion revealed that IL-2, IL-4, IL-5 and IL-10 were clearly linked in LN, but in GR cultures IL-2 was not associated with IL-4 and IL-10 production. This is consistent with the proposed model in which precursor Th2 cells express IL-2 during maturation, but subsequently reduce IL-2-producing capacity as they mature into effector or memory cells [31]. IL-10 neutralization caused strong augmentation of IFN production without concomitant increases in IL-2, suggesting the presence of a mature effector or memory Th1 population that is subject to IL-10-mediated regulation [31].

Finally, our analysis of GR macrophage TNF revealed that IL-4 and IFN may participate in different aspects of egg GR formation. As noted above, IL-4 was required for maximum cellularity, indicating a recruitment role for this cytokine in accord with models of cutaneous inflammation using polyclonal and monoclonal T cells [32,33]. Moreover, despite initial reports suggesting that IL-4 was anti-inflammatory [34], *in vitro* studies indicate a number of potential proinflammatory functions of IL-4, such as induction of vascular cell adhesion molecule-1 (VCAM-1) expression and chemokine synthesis [35,36]. Interestingly, despite its restraining effect on GR size, IFN was required for optimum TNF-producing capacity, presumably due to its macrophage-activating function [37]. Depletion of IL-4 also decreased TNF-producing capacity. This effect was surprising, since IL-4 was reported to down-regulate TNF expression *in vitro* [38]. However, our result may reflect the recruitment role of IL-4 *in vivo*, since recent studies suggest that IL-4 is required for local accumulation of Th1 cells [33]. Consequently, depletion of IL-4 would indirectly reduce TNF production by reducing the presence of potential IFN-producing cells.

In conclusion, our studies reveal a dynamic cross-regulatory cytokine network that is active during the secondary response to *S. mansoni* eggs, and provide novel information regarding local and regional cytokine participation.

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