

# Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naïve CD4<sup>+</sup> T cells

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A key feature of nematode infection is a bias towards a type 2 immune response. To investigate the role that antigen-presenting cells (APC) may play in promoting this bias, we used adherent peritoneal exudate cells (PEC) recruited in response to the filarial nematode *Brugia malayi*, to stimulate naïve T cells from pigeon cytochrome c (PCC)-specific TCR transgenic (PCC-tg) mice. Although the proliferation of PCC-tg T cells was inhibited by parasite-induced PEC during primary stimulation, they proliferated normally upon secondary stimulation and were not rendered anergic. However, PCC-tg T cells primed by suppressive APC differentiated into IL-4-producing Th2 cells upon secondary stimulation instead of IFN- $\gamma$ -producing Th1 cells, as has been previously described. Studies with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled cells indicated that Th2 differentiation was associated with the inhibition of (or failure to stimulate) IFN- $\gamma$  production during primary stimulation. Interestingly, blocking antibodies against TGF- $\beta$  (but not IL-10) restored the differentiation of IFN- $\gamma$ -producing Th1 cells. Identical results with CFSE-labeled cells were obtained using purified IL-4-dependent F4/80<sup>+</sup> macrophages. These data indicate that T cells exposed to parasite-induced alternatively activated macrophages are driven towards Th2 differentiation. This may be an important factor in the Th2 bias that accompanies nematode infection.

**Key words:** Helminth / Antigen-presenting cell / Naïve T cell / Macrophage / Transforming growth factor- $\beta$

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## 1 Introduction

Understanding why helminth infection induces type 2 cytokines has been a major goal for parasite immunologists. Since the cytokine environment is considered the most important factor influencing the differentiation of naïve T cells [1, 2], the source of early IL-4 production during helminth infections has been of particular interest [3, 4]. However, many other factors can influence the differentiation of naïve T cells, including altered peptide ligands, antigen dose and co-stimulatory molecules (reviewed by Constant and Bottomly [5]). Most recently, it has been proposed that the type of APC and its previous environmental exposure could also play an important role in influencing T cell differentiation [6, 7]. In this report, we show that a macrophage-rich adherent PEC population recruited by *Brugia malayi* can induce Th2

differentiation in the absence of detectable levels of IL-4, via a mechanism that involves TGF- $\beta$ .

*B. malayi* is a parasitic nematode that causes the human disease lymphatic filariasis. Apart from a type 2 cytokine profile, another key feature of filariasis is the presence of hyporesponsive, or anergic, T cells in the infected host [8–10]. We have previously reported that implantation of *B. malayi* into the peritoneal cavity of mice induces a strongly anti-proliferative APC in that site [11, 12]. This phenomenon is host IL-4 dependent, since the adherent PEC from *B. malayi*-implanted IL-4-deficient mice do not inhibit proliferation [12]. However, the mechanism of suppression *in vitro* is not via IL-4 and cannot be reversed by blocking antibodies against IL-4 [12]. The suppressive PEC can process and present antigen and stimulate antigen-specific IL-4 production by the Th2 clone D10.G4 [11]. However, they actively inhibit proliferation via a contact dependent mechanism (unpublished data). Based on the model of anergy induction proposed by Schwartz and Jenkins, linking cell division with clonal anergy [13, 14], we hypothesized that stimulation of naïve T cells by these anti-proliferative APC could lead to the development of anergic, or hypo-responsive cells.

[1 19832]

**Abbreviations:** PCC: Pigeon cytochrome c PCC-tg: Pigeon cytochrome-specific TCR transgenic CFSE: Carboxyfluorescein diacetate succinimidyl ester

Additionally, to explore the possibility that these suppressive APC affect T cell differentiation, we investigated their impact on the differentiation of a clonal population of naïve CD4<sup>+</sup> lymphocytes from pigeon cytochrome c (PCC)-specific TCR transgenic (PCC-tg) mice.

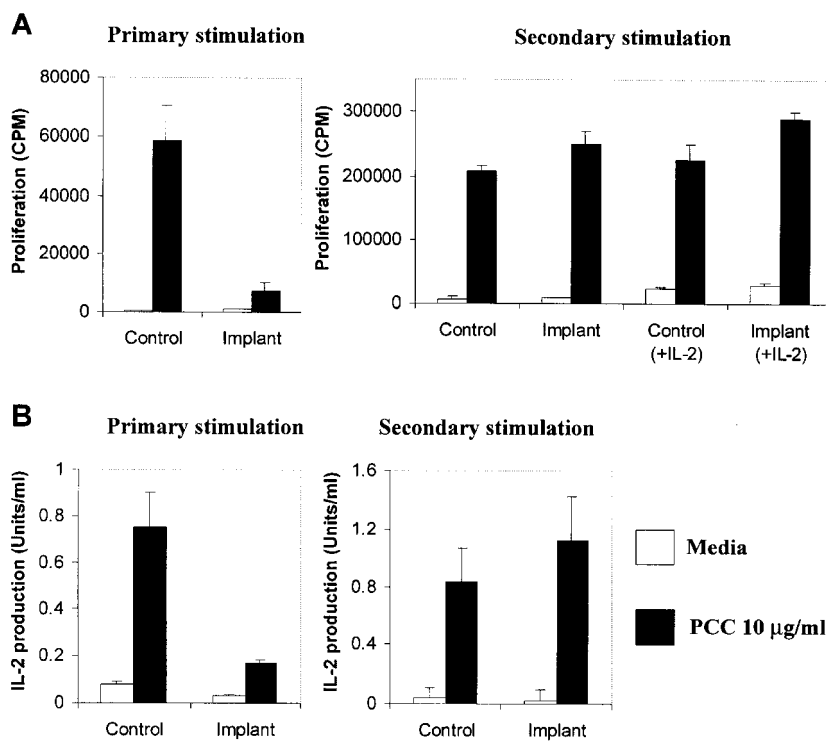
## 2 Results and discussion

### 2.1 Suppressive APC inhibit proliferation of naïve CD4<sup>+</sup> T cells but do not induce anergy

To investigate whether stimulation of naïve T cells with anti-proliferative APC could induce anergy, CD4<sup>+</sup> T cells were purified from mice expressing a transgenic TCR specific for the PCC peptide (88–104) with I-E<sup>k</sup>. These naïve CD4<sup>+</sup> cells were stimulated with either adherent PEC taken from control mice or from mice implanted with *B. malayi*. CD4<sup>+</sup> T cells proliferated strongly in response to PCC protein (10 µg/ml) when presented on control PEC, but proliferated poorly in response to stimulation by implanted PEC (Fig. 1A). IL-2 production was

also severely abrogated on primary stimulation (Fig. 1B). In a parallel experiment, suppressed CD4<sup>+</sup> T cells were removed after 48 h and rested for 72 h before re-purification and stimulation. These cells proliferated normally in response to secondary stimulation with PCC and irradiated splenocytes (Fig. 1A). IL-2 production was also fully restored (Fig. 1B).

This recovery of proliferative ability was surprising given that Schwartz and Jenkins have proposed that anergy is induced when signal 1 (TCR engagement of the peptide-MHC complex) is encountered in the absence of proliferation and IL-2 production [13, 14]. Based on this model, Powell et al. [15] showed that inhibiting proliferation of T cells with rapamycin during stimulation also leads to anergy even in the presence of co-stimulation. Our data show no induction of anergy despite profound inhibition of cell division and IL-2 production in naïve T cells during primary stimulation. Since anergy studies have primarily used established T cell clones [13, 16], our results support the observations of Hayashi et al. [17], who proposed that naïve T cells are resistant to anergy induction.



**Fig. 1.** Suppressive APC block proliferation but do not induce anergy. Purified CD4<sup>+</sup> cells from splenocytes of PCC-specific TCR transgenic mice were stimulated with adherent PEC from control or parasite-implanted animals and 10 µg/ml PCC protein. Proliferation (A) was measured by [<sup>3</sup>H]thymidine incorporation and IL-2 production (B) was measured with the NK bioassay after 48 h. Stimulated cells were also rested for 72 h, re-purified with anti-CD4 magnetic beads and restimulated with PCC and irradiated normal splenocytes for another 48 h (secondary stimulation). The addition of exogenous IL-2 (10 U/ml) had no effect on proliferation of restimulated cells (A).

## 2.2 Suppressive APC induce Th2 differentiation of naïve CD4<sup>+</sup> cells

It has been reported in several systems [18–20] that upon secondary stimulation, CD4<sup>+</sup> T cells derived from TCR transgenic mice will develop into Th1 cells and produce high levels of IFN- $\gamma$  and little IL-4, if primed in the absence of any exogenous cytokine. The addition of IL-4 during primary stimulation stimulates Th2 differentiation, leading to the production of high levels of IL-4 and little IFN- $\gamma$  [18]. We observed an identical pattern of T cell differentiation, when irradiated splenocytes were used as APC to stimulate the PCC-tg T cells (Fig. 2A). However, PCC-tg T cells primed by parasite-derived APC (in the absence of exogenous cytokines) also produced high

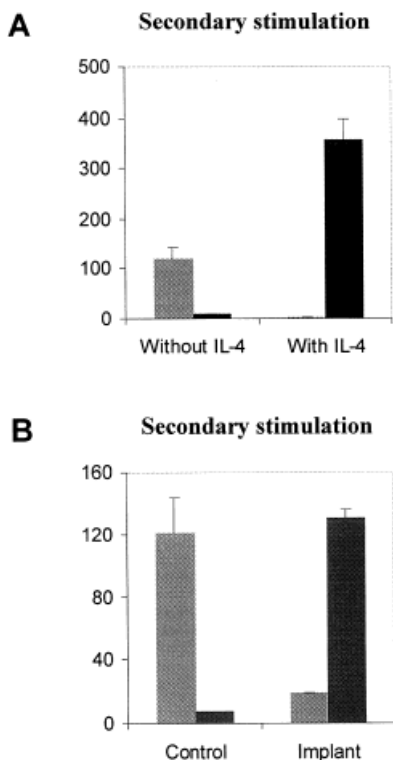
levels of IL-4 upon secondary stimulation. This was in contrast to CD4<sup>+</sup> T cells primed with control PEC, which as expected produced high levels of IFN- $\gamma$  (Fig. 2B). The mechanism by which Th2 differentiation is induced in this system did not appear to be mediated via IL-4 secretion, since IL-4 produced by PEC was generally undetectable (<5 pg/ml) in both control and implanted mice. Indeed, when very low levels of IL-4 were detected, control PEC produced equal if not greater levels of IL-4 than implant PEC (e.g. control PEC 27.8 $\pm$ 11.7 pg/ml, implant PEC 16.1 $\pm$ 10.4 pg/ml).

## 2.3 Relationship between cytokine production and proliferation

An interesting relationship between cellular proliferation and cytokine production has recently been demonstrated [21, 22]. Whereas IFN- $\gamma$  production can be stimulated without cell division, IL-4 production can only be detected after at least three rounds of cell division [21]. We therefore investigated whether parasite-derived APC could stimulate PCC-tg T cells to produce IL-4 in the absence of proliferation (during primary stimulation), by staining the T cells with the proliferation marker carboxy-fluorescein diacetate succinimidyl ester (CFSE) before primary stimulation with antigen and suppressive APC. IL-4 and IFN- $\gamma$  production were assessed by intracellular cytokine staining.

We found that naïve T cells primed with control PEC underwent up to nine rounds of cell division (in 72 h) (Fig. 3A) and produced high levels of IFN- $\gamma$  as they divided (Fig. 3B). In contrast, suppressive PEC-primed T cells went through very few rounds of cell division in the same period of time (Fig. 3A). In some experiments, they failed to divide altogether (data not shown). Furthermore, there were no IFN- $\gamma$ -producing cells among the T cells that have undergone three to four cell divisions (Fig. 3B) so that suppression of IFN- $\gamma$  appears more profound than proliferative suppression. No IL-4-producing cells were observed from T cells stimulated by either control or implant PEC (Fig. 3B).

These data suggest that the shift to Th2 differentiation we observe is more likely due to the inhibition of IFN- $\gamma$  rather than the induction of IL-4-producing T cells during primary stimulation. This is consistent with observations by Cua and Stohlmans [23], which suggest that reduced IL-12 production by a subpopulation of macrophages, could be associated with type 2 responses [23]. IL-12 production by monocyte/macrophages can be down-regulated by a number of factors including engagement of CD47 [24] and ligation of the Fc $\gamma$ , complement, or scavenger receptors [25]. Alternatively, the suppressive



**Fig. 2.** Suppressive APC induce Th2 differentiation. (A) PCC-specific TCR transgenic CD4<sup>+</sup> cells were stimulated with 10  $\mu$ g/ml PCC protein and irradiated splenocytes in the absence or presence of IL-4 (200 pg/ml). After 5 days, primed cells were washed and restimulated with fresh irradiated splenocytes; 48 h later, supernatants were harvested and tested for IL-4 (■, pg/ml) and IFN- $\gamma$  (▒, U/ml) production. (B) Naïve T cells were primed with control or parasite implanted PEC without additional cytokines (as described in Fig. 1). After 5 days, primed cells were restimulated with PCC and fresh irradiated normal splenocytes; and 48 h after secondary stimulation, supernatants were harvested and tested for IL-4 (■, pg/ml) and IFN- $\gamma$  (▒, U/ml) production.

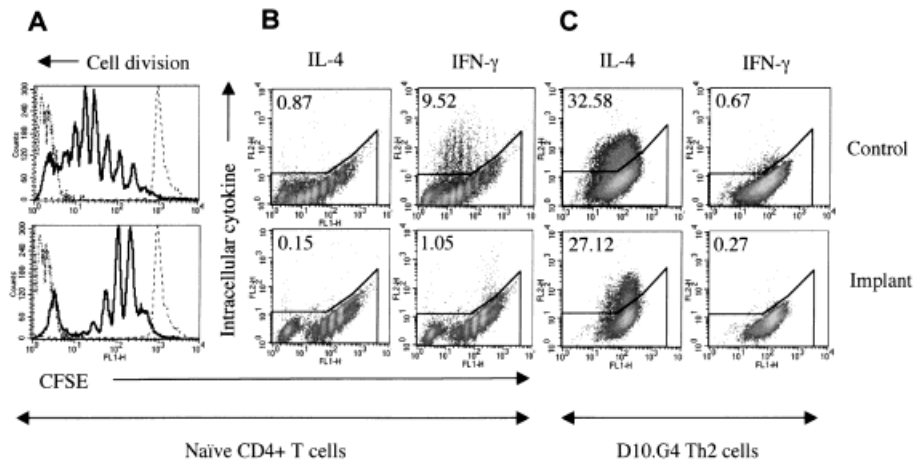


Fig. 3. Analysis of cell division and cytokine production during primary stimulation. Naive CD4<sup>+</sup> T cells and the Th2 clone D10.G4 were labeled with CFSE before stimulation with antigen and adherent PEC from control or parasite implanted mice. After 72 h, the nonadherent T cells were harvested and stained for intracellular cytokine (IL-4 and IFN- $\gamma$ ). The numbers in (B) and (C) represent the percentage of cytokine positive cells. (A) Cell division of naive T cells stimulated with PCC and control PEC or implant-derived PEC. (B) Intracellular IL-4 and IFN- $\gamma$  staining of dividing CD4<sup>+</sup> transgenic T cells. (C) As a positive control for IL-4 staining, the Th2 clone D10.G4 was also stimulated with conalbumin and control or implant PEC. Intracellular IL-4 and IFN- $\gamma$  was assessed.

APC may be producing down-regulatory cytokines that directly inhibit IFN- $\gamma$  production [26–28].

#### 2.4 TGF- $\beta$ is involved in inhibition of IFN- $\gamma$ -producing T cells

IL-10 and TGF- $\beta$  are cytokines commonly associated with the capacity to down-regulate IFN- $\gamma$  [29, 30] as well as inducing a Th2 differentiation bias [27, 31, 32]. To determine whether these molecules were involved in the inhibition of IFN- $\gamma$  production or proliferative suppression of naive T cells, we used blocking antibodies against IL-10 and TGF- $\beta$  to see if the observed phenomena could be reversed (Fig. 4). Consistent with the fact that IL-10 was undetectable in primary stimulation assays (<50 pg/ml), we found that blocking antibodies against IL-10 did not have any significant effect on naive T cells primed by parasite-induced APC (Fig. 4C). However, blocking antibodies against TGF- $\beta$  doubled the number of IFN- $\gamma$ <sup>+</sup> cells stimulated by the suppressive PEC (Fig. 4D), reaching numbers which were indistinguishable from control PEC-stimulated T cells. Upon secondary stimulation, IFN- $\gamma$  production by T cells primed with suppressive PEC and anti-TGF- $\beta$  was also indistinguishable from those primed with control PEC (Fig. 5A), and was much enhanced compared to priming with suppressive PEC alone. IL-4 production was also significantly reduced as a result of primary stimulation with anti-TGF- $\beta$  antibodies (Fig. 5B). This suggests that

TGF- $\beta$  plays a role in the induction of IL-4 production in naive T cells stimulated by suppressive PEC, as well as inhibiting their production of IFN- $\gamma$ .

Although these experiments suggested that TGF- $\beta$  was involved in Th2 induction by suppressive PEC, blocking

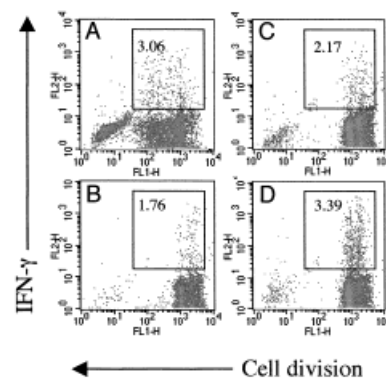
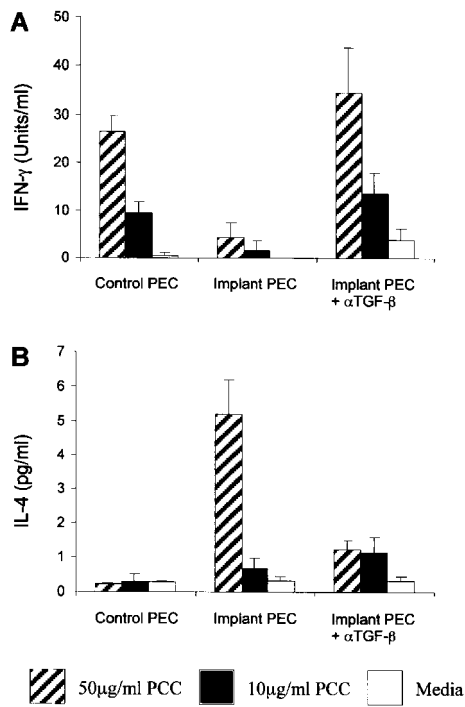


Fig. 4. TGF- $\beta$  is involved in inhibition of IFN- $\gamma$  by implant PEC, but not inhibition of proliferation. Naive CD4<sup>+</sup> T cells were labeled with CFSE before stimulation with antigen and PEC from control mice (A) or parasite-implanted mice (B–D). Media (A, B), neutralizing anti-IL-10 (C) or neutralizing anti-TGF- $\beta$  (D) were added to the *in vitro* cultures. Blocking antibodies had no effect on T cells stimulated by control PEC (data not shown). After 72 h, the naive T cells were harvested and stained for intracellular IL-4 (undetectable, data not shown) and IFN- $\gamma$ . Numbers within gates represent the percentage of IFN- $\gamma$ <sup>+</sup> cells.



**Fig. 5.** TGF- $\beta$  is involved in inducing Th2 differentiation. Naïve T cells were primed (as described in Fig. 1 and 2) with control PEC, parasite-implanted PEC alone and parasite-implanted PEC with blocking antibodies against TGF- $\beta$ . After 5 days, primed cells were restimulated with 10 or 50  $\mu$ g/ml PCC and fresh irradiated splenocytes. At 72 h after secondary stimulation, supernatants were harvested and tested for IL-4 and IFN- $\gamma$  production.

antibodies against TGF- $\beta$  did not reverse the proliferative suppression of naïve T cells (Fig. 4D). The observation that antibodies against TGF- $\beta$  and IL-10 do not reverse proliferative suppression is consistent with our previous work using Th2 clones and transformed cell lines [11, 12]. The observation that antibodies against TGF- $\beta$  could reverse IFN- $\gamma$  inhibition, but not proliferative suppression, also suggests that there is no direct relationship between these two phenomena.

There remain many unanswered questions about the role of TGF- $\beta$  in our system, especially since its role in naïve T cell differentiation is extremely complex with conflicting data from different experimental systems [30]. While our observations are consistent with reports that TGF- $\beta$  is associated with Th2 development and the inhibition of IFN- $\gamma$  [28, 32–34], it is in contrast with reports suggesting that TGF- $\beta$  inhibits Th2 differentiation [35–37]. Thus, the effects of TGF- $\beta$  vary depending on a variety of factors (e.g. cytokine concentration [38] and genetic background [39]), and cannot be generalized beyond a particular system. Furthermore, it is uncertain in our system

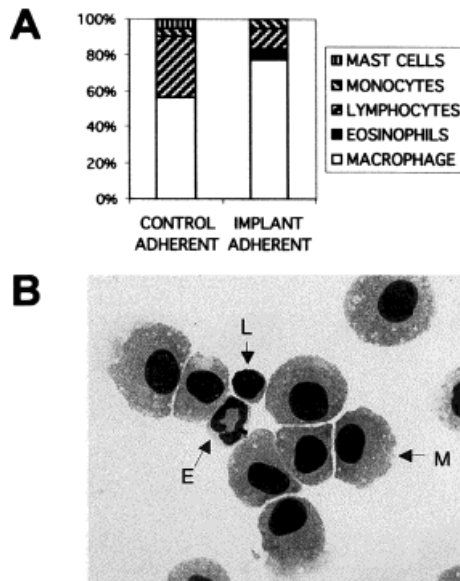
whether TGF- $\beta$  acts directly on naïve T cells or has an indirect effect. TGF- $\beta$  production by parasite-derived APC could be directly influencing IFN- $\gamma$  production by inhibiting the IL-12 responsiveness of the naïve T cells as a result of down-regulating IL-12R expression [33]. However, TGF- $\beta$  has also been shown to affect T cell differentiation by modulating the phenotype of APC rather than acting directly on T cells [20, 40]. PEC pulsed with TGF- $\beta$  can induce IL-4 production by DO11.TCR transgenic naïve T cells even during primary stimulation [20, 40]. The stimulation of IL-4 appeared to be a result of CD40 up-regulation, as well as inhibition of IL-12 production [20]. We are currently investigating whether TGF- $\beta$  is acting directly or indirectly in our system.

## 2.5 Effect of parasite recruited macrophages on naïve T cells

We have previously described an almost tenfold increase in total cell numbers ( $3.1 \pm 0.8 \times 10^7$ ) recovered from the peritoneal cavity of parasite-implanted mice, in comparison to control mice ( $3.8 \pm 1.1 \times 10^6$ ). Furthermore, parasite-implanted mice had a marked reduction in mast cells and an increase in both macrophages and eosinophils [12]. The adherent component of the PEC populations, which are cultured with the naïve T cells during primary stimulation, show a similar profile (Fig. 6).

F4/80<sup>+</sup> macrophages have been recently identified as the PEC cell type directly responsible for proliferative suppression in implanted mice (submitted for publication). These IL-4-dependent macrophages represent the majority of the suppressive PEC population (around 80%) and will present antigen and stimulate IL-4 production by the Th2 clone D10.G4, while inhibiting proliferation (submitted for publication). To examine the possibility that macrophages are responsible for the observed effect on naïve T cells, F4/80<sup>+</sup> cells were purified from the PEC population of control and parasite implanted mice, and used to stimulate CFSE stained naïve T cells (Fig. 7). Whereas F4/80<sup>+</sup> cells from control animals stimulated cell division and IFN- $\gamma$  production by naïve T cells, parasite recruited F4/80<sup>+</sup> cells suppressed proliferation and did not stimulate IFN- $\gamma$ -producing cells (Fig. 7). This data demonstrates that it is the macrophage population that is responsible for the observed effects on IFN- $\gamma$  production. This suggests that alternatively-activated macrophages influence naïve T cell differentiation by failing to stimulate, or inhibiting, IFN- $\gamma$  production, perhaps permitting a Th2 default pathway of differentiation.

Recent reports have described specific subsets of dendritic cells that differ in their capacity to stimulate the differentiation of naïve T cells [7, 27, 31]. However, we are

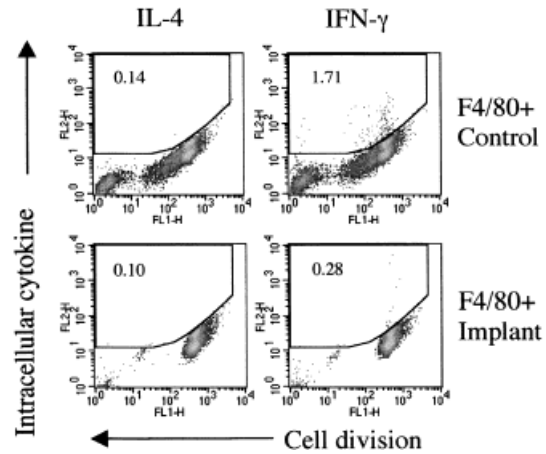


**Fig. 6.** Adherent peritoneal cell populations in control and parasite-implanted mice. Adherent cells were removed non-enzymatically from tissue culture wells with a cell scraper, after the nonadherent cells had been washed away with medium. The cell composition of adherent PEC was determined by microscopy of cytocentrifuge preparations stained with Diff Quik (A). Data shown are mean percentage of mast cells, eosinophils, lymphocytes, macrophages and unidentified monocytes, from three cytocentrifuge preparations per sample. Cells ( $n=500$ ) were counted from randomly selected fields per cytocentrifuge preparation. (B) An example of PEC recruited by *B. malayi*. L; lymphocyte, E; eosinophil, M; macrophage.

unaware of any reports suggesting that macrophages can also differ in their capacity to stimulate naïve T cell differentiation, although this has been proposed by Goerdts et al. [6]. Indeed, macrophages are expected to stimulate a Th1 response [41]. Our observations imply that macrophages, like dendritic cells, might also differ in their capacity to stimulate T cell differentiation.

### 3 Concluding remarks

It is still uncertain how helminths induce biased type 2 immune responses. In this study, we show that recruitment of alternatively activated macrophages by a parasitic nematode could play a significant role in inducing Th2 differentiation of naïve T cells and biasing the immune response. Interestingly, Th2 differentiation of naïve T cells appears to correlate with inhibition or failure to induce IFN- $\gamma$  production during primary stimulation, rather than up-regulation of early IL-4 release. Furthermore, TGF- $\beta$  appears to be involved in the inhibition of IFN- $\gamma$  production although its exact role has yet to be determined.



**Fig. 7.** F4/80<sup>+</sup> macrophages from the implant PEC population inhibit IFN- $\gamma$ . Naïve CD4<sup>+</sup> T cells were labeled with CFSE before stimulation with antigen and F4/80<sup>+</sup> purified cells from control or parasite-implanted mice. After 72 h, the naïve T cells were harvested and stained for intracellular IL-4 and IFN- $\gamma$ . Numbers in gate represents percentage of IFN- $\gamma$ <sup>+</sup> cells.

## 4 Materials and methods

### 4.1 Parasites and mouse infection

*B. malayi* adult parasites were obtained from infected jirds purchased from TRS laboratories (Athens, GA). Adult worms were removed from the peritoneal cavity and washed in RPMI supplemented with 50  $\mu$ g/ml gentamicin (RPMI wash). For all experiments, mice used were 6–8-week-old CBA/Ca males. Mice were surgically implanted intraperitoneally (i.p.) with six live adult *B. malayi* females and 3–6 weeks later mice were killed by cardiac puncture and PEC were harvested by thorough washing of the peritoneal cavity with 15 ml of RPMI wash.

### 4.2 Transgenic T cells and T cell lines

All *in vitro* cultures were carried out in RPMI 1640 medium supplemented with 2 mM glutamine, 0.25 U/ml penicillin, 100  $\mu$ g/ml of streptomycin, 5  $\mu$ M 2-mercaptoethanol and 10% FCS (RPMI complete). The Th2 cell clone, D10.G4 [42] was maintained in culture as previously described [11]. Naïve CD4<sup>+</sup> lymphocytes were obtained from splenocytes of mice expressing transgenic TCR for PCC peptide 88–104/I-E<sup>K</sup> (B10.AxB10 F1) [18]. CD4<sup>+</sup> lymphocytes (>95% pure) were isolated with directly conjugated L3T4 beads and either MS<sup>+</sup> or VS<sup>+</sup> columns in conjunction with MiniMacs or VarioMacs magnet (Miltenyi Biotec).

### 4.3 Antigen-specific stimulation of T cells

For primary stimulation studies,  $1 \times 10^5$  PEC from control or parasite-implanted mice were plated out on flat-bottom 96-well plates for 2–3 h at 37°C. The nonadherent cells and microfilariae were then removed by washing with complete medium. The adherent PEC were used to stimulate  $1 \times 10^5$  purified naïve CD4<sup>+</sup> T cells, with 10 µg/ml PCC protein. Supernatants were harvested after 48 h for cytokine assays (see below) and proliferation was measured by [<sup>3</sup>H]thymidine incorporation. For two stage assays,  $1 \times 10^6$  PEC were adhered to 24-well plates, washed and used to stimulate  $1 \times 10^6$  naïve T cells (at 10 µg/ml PCC protein). After 48-h coculture, nonadherent T cells were aspirated from adherent PEC and allowed to expand/rest for 72 h in complete media before being repurified for CD4<sup>+</sup> cells as above. In secondary cultures,  $1 \times 10^5$  repurified T cells were stimulated with  $5 \times 10^5$  irradiated splenocytes and 10 or 50 µg/ml PCC protein. After 48 h, proliferation and cytokine production were assayed as described for primary stimulation. Neutralizing anti-TGF-β [43] (clone 1D11.16) was the kind gift of the Celltrix Corporation, Santa Clara, CA. Neutralizing anti-IL-10 (clone JES5-2A5) was purchased from PharMingen, San Diego, CA. The antibodies were added to the primary stimulation cultures at the recommended concentrations (25 µg/ml anti-TGF-β and 5 µg/ml anti-IL-10).

### 4.4 Cytokine assays

IL-2 and IL-4 production were measured with the IL-2/IL-4-responsive NK cell line, as described previously [11]. Briefly,  $1 \times 10^4$  NK cells were added to 10 µl of cell culture supernatant in the presence of saturating anti-IL-2 (S4B6) or anti-IL-4 (11B11) antibody to distinguish between the two cytokines responsible for proliferation. Proliferation data was converted to concentrations with standard curves of recombinant IL-4 and IL-2 (Sigma). IFN-γ and IL-10 was measured by capture ELISA, with reference to standard curves of known amounts of recombinant (r)IFN-γ (Sigma) and rIL-10 (Genzyme). IFN-γ was measured using R46A2 (ATCC) as capture Ab and biotinylated rat anti-mouse IFN-γ monoclonal XMG1.2 (PharMingen). IL-10 was measured using JES5-2A5 (PharMingen) as capture Ab and biotinylated rat anti-mouse IL-10 clone SXC-1 (PharMingen). Avidin-alkaline phosphatase (Sigma) was used for detection.

### 4.5 CFSE and intracellular cytokine staining

A modification of Lyons and Parish's technique for CFSE staining was used [21, 44]. Briefly,  $1 \times 10^7$  cells/ml were incubated with 10 µM CFSE (Molecular Probes) in PBS for 8 min at room temperature. Staining was stopped with an equal volume of FCS, and the cells were washed three times with complete RPMI. Stained naïve T cells were primed by PEC (as described above) for 72 h before being harvested and stained for intracellular cytokines using the Cytotfix/Cyto-

perm Plus™ kit (PharMingen) according to the manufacturer's instructions. Briefly, GolgiStop was added to the culture at 1:1500 dilution together with 50 ng/ml PMA and 500 ng/ml ionomycin and incubated for 4 h at 37°C. Nonadherent cells were harvested and fixed in Cytotfix/Cytoperm solution before being stained with anti-IL-4 (11B11, PharMingen), anti-IFN-γ (XMG1.2, PharMingen) or control Ig-PE conjugates (PharMingen) in Perm/Wash solution. After staining, cells were analyzed by flow cytometry using a Becton Dickinson FACScan and Cell Quest Software. All data plots shown represent activated lymphocytes, gated by forward and side light scatter.

### 4.6 Cell sorting and analysis

Before magnetic bead cell purification, PEC were passed through a 70-µm cell strainer and purified by centrifugation over Histopaque (Sigma) to remove any microfilariae. PEC were then sorted with MS+ or VS+ columns according to the manufacturers instructions (Miltenyi Biotec). F4/80<sup>+</sup> cells were purified with biotin conjugated F4/80 (rat IgG2b; Caltag) and streptavidin microbeads (Miltenyi Biotec).

### 4.7 Phenotype of adherent PEC

PEC from control and implanted animals were incubated in 24-well plates for 2–3 h, after which the nonadherent cells were removed by washing with complete medium and the adherent cells were removed non-enzymatically with a cell scraper. Cyto-centrifuge preparations of the adherent PEC ( $1 \times 10^5$  cells/slide) were air-dried and fixed in methanol before staining with Diff-Quik (Dade Diagnostics, Unterschleissheim, Germany). The proportion of macrophages, lymphocytes, mast cells, and eosinophils was determined by morphological examination of at least 500 cells in randomly selected fields using an Olympus BH2 microscope.

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