Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact

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The cytokine microenvironment is thought to play an important role in the generation of immunoregulatory cells. Nematode infections are commonly associated with Th2 cytokines and hyporesponsive T cells. Here we show that IL-4-dependent macrophages recruited *in vivo* by the nematode parasite *Brugia malayi* actively suppress the proliferation of lymphocytes on co-culture *in vitro*. These alternatively activated macrophages block proliferation by cell-to-cell contact, implicating a receptor-mediated mechanism. Further, the proliferative block is reversible and is not a result of apoptosis. Suppressed cells accumulate in the G1 and G2/M phase of the cell cycle. Interestingly, the G1 and G2/M block correlates with increased levels of Ki-67 protein, suggesting a mechanism that affects degradation of cell cycle proteins. We also show that, in addition to lymphocyte cell lines of murine origin, these suppressive cells can inhibit proliferation of a wide range of transformed human carcinoma lines. Our data reveal a novel mechanism of proliferative suppression induced by a parasitic nematode that acts via IL-4-dependent macrophages. These macrophages may function as important immune regulatory cells in both infectious and noninfectious disease contexts.

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1 Introduction

Understanding mechanisms that regulate cellular proliferation is a fundamental goal of cell biology, developmental biology, oncology, as well as immunology. In immunological systems, APC and T cells can both adopt an important role in regulating or suppressing the proliferation of immune cells and thereby modulate immune response expansion and development. Prominent $CD4^+$ regulatory T subsets [1–3] have been shown to inhibit the proliferation of other T cells via cytokines such as IL-10 and TGF-β [4, 5]. Most recently, a direct suppressive mechanism dependent on cell-to-cell contact between regulatory and responder T cells has been proposed [1, 6, 7].

Among APC, the most well characterized suppressive (or regulatory) cells are macrophages "classically activated" by Th1 cytokines (such as IFN-γ). By the release of NO [8–10] these cells suppress cellular proliferation by multiple mechanisms including the inhibition of DNA synthesis and its rate-limiting enzyme, ribonucleotide reductase [11]. In addition to activation by pro-inflammatory cytokines, macrophages can be alternatively activated by type 2 cytokines such as IL-4 and IL-13 [12, 13]. These cells have been less well characterized than classically activated macrophages but appear to have anti-inflammatory roles and suppress T cell proliferation via cytokines such as TGF-β and IL-10 [14].

We have previously found that a parasitic nematode, *Brugia malayi*, when implanted into the peritoneal cavity of mice, generates host cells that inhibit proliferation of immune cells [15]. Significantly, the recruitment or induction of these suppressive cells is dependent on host production of the Th2 cytokine IL-4, as they are not found in mice genetically deficient in IL-4 [16]. This finding is consistent with the induction of type 2 responses by *B. malayi*, a characteristic feature of infection with nematode parasites [17]. Interestingly, IL-10 does not play an important role either in the recruitment of these suppressive cells or their ability to inhibit proliferation, since suppressive cells can be generated in IL-10-deficient mice and proliferative suppression cannot be reversed by blocking antibodies against IL-10 [16].

In this study we set out to both establish the identity of the suppressive cell and to elucidate the mechanism of

The first two authors contributed equally to the work.

Abbreviation: CFSE: Carboxyfluorescein diacetate succinimidyl ester **TUNEL:** Terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nuck end labeling **Implant PEC:** PEC from parasite implanted mice

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proliferative suppression. Although initial work implicated eosinophils and not F4/80$^+$ macrophages as the suppressive population [16], more recent experiments suggested that the original findings on this issue were misplaced [18]. Here we establish that F4/80$^+$ macrophages are the suppressive population, and exclude a suppressive role for eosinophils, since IL-5-deficient mice capably generated anti-proliferative cells. The requirement for the type-2 cytokine IL-4 places these macrophages in the category of "alternatively activated" macrophages that have been suggested to play an important role as anti-inflammatory cells [14]. The nematode-induced macrophages showed several novel properties not previously described for anti-inflammatory macrophages. Instead of soluble cytokines, proliferative suppression occurred via cell-to-cell contact, suggesting a receptor-mediated mechanism. Furthermore, suppressed cells were not simply blocked at the G1/G0 stage of the cell cycle but also at the G2/M stage. Finally, the proliferation of a wide range of transformed human cell lines was also inhibited by these macrophages. These data suggest that alternatively activated macrophages induced by a Th2-driving infection can regulate proliferation of a broad range of cell types via a novel contact-mediated mechanism.

2 Results

2.1 Proliferative suppression is mediated by IL-4-dependent macrophages

Adherent PEC from mice implanted with *B. malayi* (implant PEC) profoundly inhibit proliferation of cloned lymphocytes lines (*e. g.* D10.G4) and transformed lymphocytes (*e. g.* HB32 and EL4) [15, 16]. The peritoneal cells recruited by *B. malayi* consist of a mixed population consisting mainly of macrophages (74.3 ± 3.5 %), eosinophils (11.7 ± 0.5 %) and lymphocytes (8.6 ± 4.7 %) [16]. Critically, IL-4-deficient mice that have reduced numbers of eosinophils fail to generate suppressive APC [16]. We therefore chose to examine the role of eosinophils in suppression by implanting *B. malayi* into IL-5-deficient mice, which are defective in eosinophil recruitment [19]. The absence of eosinophils recruited into the peritoneal cavity by *B. malayi* in IL-5-deficient mice was confirmed by cytospin analysis of the PEC population (data not shown). However, PEC from IL-5-deficient mice exhibited anti-proliferative activity equivalent to that of control C57BL/6 mice while IL-4 deficient PEC were unable to suppress proliferation (Fig. 1 A). We therefore concluded that eosinophils do not play a significant role in the suppressive mechanism. To ensure that a small population of CD11c$^+$ dendritic cells did not mediate suppression, we used magnetic sorting to purify CD11c$^+$ cells from PEC, and found that CD11c$^+$ cells were not suppressive (data not shown).

This provided strong evidence that an IL-4-dependent (but IL-5-independent) cell type was directly responsible for the proliferative suppression. Although earlier studies had provided evidence that macrophages were not the suppressive cell [16], these data necessitated a re-evaluation of the role of macrophages. We thus purified F4/80$^+$ cells from *B. malayi*-implanted mice using magnetic beads and found that these cells suppress proliferation of EL4, HB32 (Fig. 1 B) and D10.G4 cells (Fig. 1 C) after 48 h of co-culture. Cytospin analysis confirmed that F4/80 purified cells were macrophages (Fig. 1 D). Furthermore, we showed that F4/80 purified macrophages from implanted mice can stimulate antigen-specific cytokine production by D10.G4 cells, while inhibiting proliferation (Fig. 1 C), repeating our original observations using unsorted adherent PEC. The lack of proliferation of D10.G4 cells is therefore not a result of defective antigen processing and presentation capabilities of the macrophages, but is instead due to an active mechanism. We thus confirmed that parasite-exposed macrophages alone can directly mediate proliferative suppression while presenting antigen and stimulating cytokine production.

2.2 Suppression is mediated by cell contact

Previous investigations have excluded the action of soluble factors commonly associated with suppressive macrophages, namely prostaglandins, NO, IL-10 and TGF-β [15, 16]. We therefore chose to determine whether the suppressive effect of parasite-recruited PEC is indeed mediated through the release of a soluble factor, or is dependent on cell-to-cell contact. For these experiments, the suppressive PEC population was separated from HB32 and D10.G4 cells by a 0.4 μm-pore membrane in a transwell culture. The anti-proliferative activity of parasite-recruited PEC was reversed by transwell separation (Fig. 2 A), indicating that suppression was mediated either by direct contact or by a very labile soluble factor. To distinguish between these possibilities, suppressive PEC were fixed with 1 % paraformaldehyde before being co-cultured with responder cells. The fixed PEC from implanted mice fully retained their ability to block proliferation of HB32, EL4 and D10.G4 cells, demonstrating that the anti-proliferative signal is effected by cell contact and does not require the release of soluble mediators (Fig. 2 B). Although a contact-dependent mechanism is sufficient for suppression, it is likely that these macrophages also produce soluble suppressive compounds, since some inhibition of proliferation could be observed across the transwell (Fig. 2 A, HB32 cells).

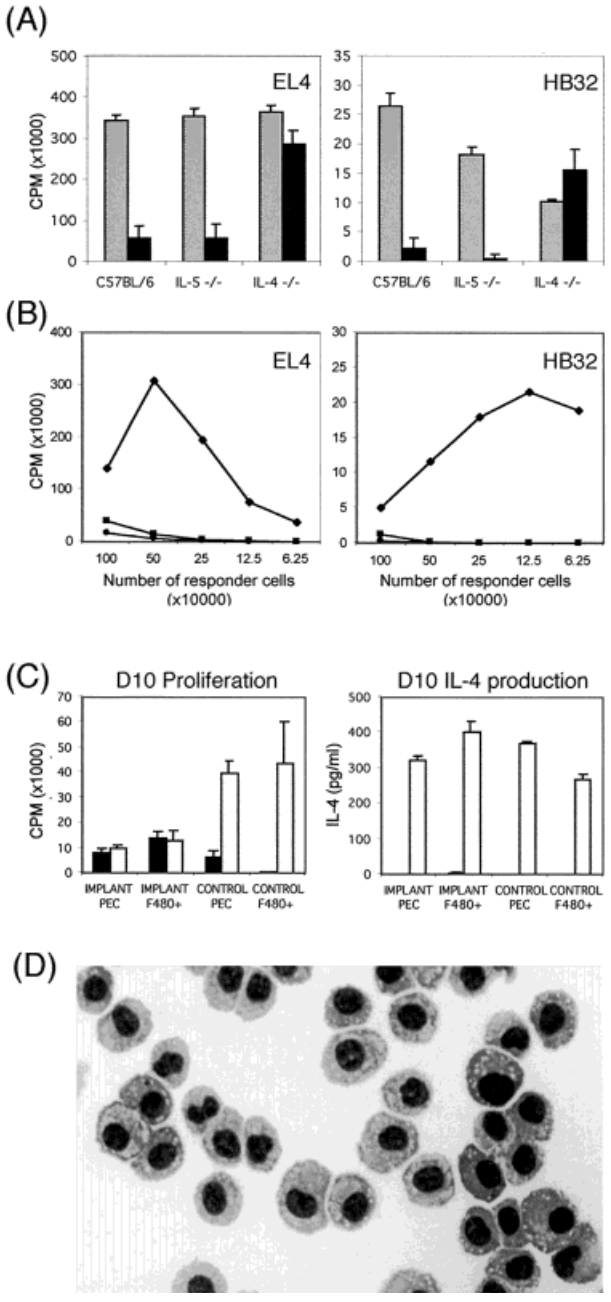

Fig. 1. IL-4-dependent macrophages suppress proliferation but stimulate cytokine production. (A) PEC from control (▨) and parasite-inplanted (■) IL-5$^{-/-}$, IL-4$^{-/-}$ and C57BL/6 mice were co-cultured with EL-4 and HB32 cells. After 48 h, proliferation was measured by [^{3}H] thymidine incorporation. Data shown are mean ± SD of five mice assayed individually. (B) F4/80$^+$ cells were purified (> 95 % pure) with magnetic beads. EL4 and HB32 cells were cultured at different cell concentrations with fixed numbers (1 × 10^5 cells per well) of control PEC (◆), implant PEC (●) and F4/80$^+$ purified cells from implant PEC (■). After 48 h, proliferation was measured by [^{3}H] thymidine incorporation. (C) D10 cells were co-cultured with control and implant PEC, as well as F480$^+$ cells purified from these PEC populations. After 48 h of co-culture with (□) or without antigen (■), IL-4 production was assayed by bioassay, and proliferation was measured by [^{3}H] thymidine incorporation. (D) Cytospin of purified F4/80$^+$ cells stained with Diff-Quik, observed at 200X magnification.

2.3 Cell cycle arrest is reversible and does not lead to apoptosis

To determine if the absence of proliferation in responder cells was a result of apoptosis induced by suppressive PEC, we assessed apoptosis in suppressed D10.G4 cells and EL4 cells by terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining. PEC alone were also assessed as a control for background levels of apoptosis. These experiments revealed that some of the parasite-recruited PEC undergo apoptosis after 48 h in culture (Fig. 3 A). However, apoptosis of D10.G4 cells and EL4 cells was not significantly increased by exposure to suppressive PEC (Fig. 3 B and C) when compared to the background levels of apoptosis observed in parasite-recruited PEC (Fig. 3 A). Apoptosis is therefore not the explanation for the reduction of proliferation.

Since the non-proliferating responder cells were not undergoing apoptosis, we asked if they could recover their ability to proliferate after being removed from the suppressive cells. D10.G4 and EL4 cells were removed from adherent suppressive PEC after 48 h co-culture, purified and proliferation measured after an additional 48 h. These experiments demonstrated that suppressed responder cells could fully recover their ability to proliferate (Fig. 4). Indeed, thymidine incorporation of recovered cells was actually elevated (Fig. 4 B), perhaps due to cell cycle synchronization by the proliferative block.

2.4 Suppressive PEC inhibit proliferation of various transformed cell lines

We had previously observed that proliferative suppression is not antigen specific, acting on mitogen-activated T cells as well as affecting transformed lymphocytes [15]. We wanted to address how wide-ranging this anti-proliferative effect was by exposing a broad panel of human tumor cells to the suppressive PEC. Strikingly, the proliferation of many human tumor cell lines was inhibited when co-cultured with suppressive PEC, in comparison to control PEC (Table 1). This indicated that the suppressive mechanism was neither species specific nor cell type specific. However, the sensitivity of the different types of carcinoma to suppression by PEC did

Table 1. Effect of suppressive PEC on human tumor cell lines[a)]

Cell line	Control PEC (cpm)	Implant PEC (cpm)	Suppression (%)	Cancer type
H358	115888	15111	87	Non-small-cell lung adenocarcinoma
HCT116	140116	19996	85.7	Colon carcinoma
HT29	55420	12832	76.8	Colon carcinoma
LoVo	180491	54441	69.8	Colon carcinoma
Colo320	251051	86639	65.4	Colon carcinoma
H2122	148989	55024	63	Non-small cell lung carcinoma
Capan-1	50261	25675	48.9	Pancreatic carcinoma
COR L24	2522	1312	48	Small cell lung carcinoma
H524	69167	42403	38.7	Small cell lung carcinoma
Panc-1	86934	54109	37.7	Pancreatic carcinoma
MiaPaCa-2	172272	135719	21.2	Pancreatic carcinoma
NGP	112649	119571	– 6.1	Neuroblastoma

a) Cancer cells (1×10^4) were cultured with adherent PEC (1×10^5) from control or implanted animals in 96-well flat-bottom plates. Proliferation was measured at 48 h by [^{3}H] thymidine incorporation and results are shown as cpm. Numbers shown are representative of at least two experiments with each cell line.

vary considerably. Colon carcinomas appeared to be more sensitive to suppression than pancreatic carcinomas, while the neuroblastoma line NGP was completely unaffected (Table 1).

2.5 Effect of suppressive PEC on the cell cycle

In previous experiments, proliferation of cells co-cultured with adherent PEC was measured by [^{3}H] thymidine incorporation after 48 h [15, 16]. To obtain a more com-

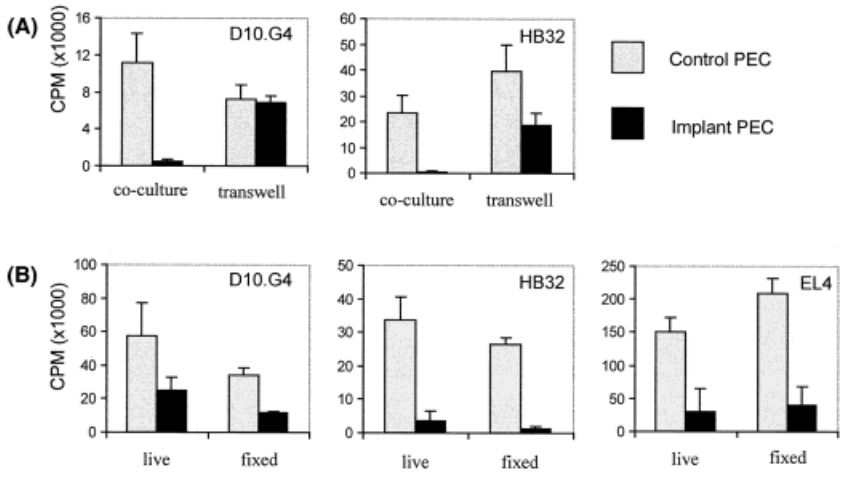

Fig. 2. Proliferative inhibition is mediated by cell-to-cell contact. (A) D10.G4 and HB32 cells were either directly co-cultured with PEC or placed in separate transwell chambers separated by a 0.4-μm membrane. To obtain sufficient cell numbers, PEC from separate animals were combined, and data are shown as mean ± SD for six wells. Data shown are representative of three experiments. Although proliferation of separated HB32 cells was slightly reduced in some experiments (as shown), there were no significant differences in other experiments. (B) Control or implant PEC were fixed with 1 % paraformaldehyde before being co-cultured with D10.G4, EL4 and HB32 cells. Proliferation was measured by [^{3}H] thymidine incorporation. Data shown are mean ± SD of individual mice assayed separately and are representative of two independent experiments.

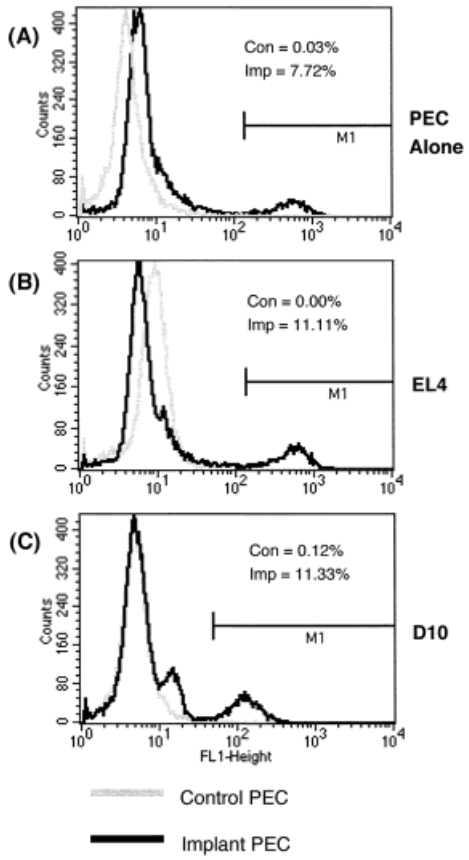

Fig. 3. Proliferative inhibition does not lead to apoptosis. Cells were TUNEL stained for apoptotic cells 48 h after stimulation/co-culture with control PEC (black line) or implant PEC (gray line). M1 represents apoptotic cells. (A) Implant PEC alone undergo some apoptosis, whereas control PEC do not. (B) EL4 and (C) D10.G4 cells had slightly elevated levels of apoptosis in comparison to implant PEC alone. Data are representative of two experiments.

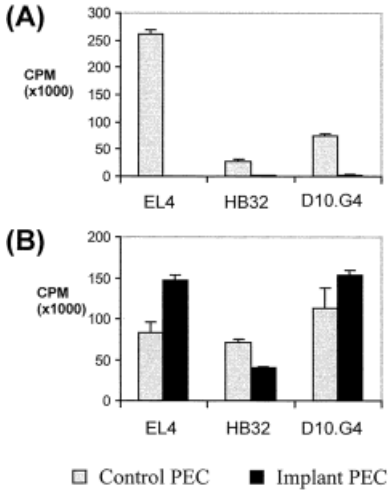

Fig. 4. Proliferative suppression is reversible. (A) Proliferation of EL4, HB32 and D10.G4 (with 50 μg/ml conalbumin) cells was measured by [^{3}H] thymidine incorporation after 48 h co-culture with control and implant PEC. (B) After 48 h exposure to PEC, nonadherent HB32, EL4 and D10 cells were purified through nylon wool columns. Recounted cells were cultured for 48 h and proliferation measured. D10 cells were restimulated with fresh APC and antigen. PEC of individual animals were combined for this experiment and results shown are mean ± SD of quadruplicate wells.

plete picture, we performed experiments to assess the effect of suppressive PEC on the cell cycle of the responder cells.

Firstly, in order to study the kinetics of the proliferative block, EL4 cells were labeled with proliferation marker dyes PKH26 or carboxyfluorescein diacetate succinimidyl ester (CFSE) before co-culture with PEC. These dyes halve in intensity with each cell division and provide the means to track cell divisions that occur after exposure to suppressive PEC [20]. In these experiments, EL4 cells cultured with suppressive PEC completed one round of cell division after 24 h, but failed to undergo a second round of cell division after 48 h (Fig. 5 B). This was also observed when CFSE-labeled D10.G4 cells were stimulated with either control or implant PEC. Whereas control PEC-stimulated D10.G4 cells had undergone up to three rounds of cell division after 48 h, suppressed D10.G4 cells had only completed one round of cell division (Fig. 5 C).

Secondly, D10.G4 and EL4 cells were stained with propidium iodide after co-culture with PEC for 48 h to identify at which point cell cycle arrest occurred. The effect of co-culture with suppressive PEC on both cell lines (in comparison with control PEC) was to increase the percentage of cells in G0/G1 and G2/M, and to reduce the number of cells in S phase (Fig. 6 A). The decrease in the percentage of S phase cells is consistent with the inhibition of thymidine incorporation in standard proliferation assays. The accumulation of suppressed cells at both G0/G1 as well G2/M phases suggested that there were two blocks on the cell cycle.

To further distinguish between cells in G0 and G1, cells were double stained with antibody against Ki-67 as well as propidium iodide. Ki-67 is usually expressed only in proliferating cells and not in quiescent G0 phase cells. Surprisingly, suppressed D10.G4 cells and EL4 cells had an increased level of Ki-67 protein in comparison to proliferating cells cultured with control PEC (Fig. 5 B and C). The accumulation of Ki-67 was evident in G1 cells (2N) as well as G2/M cells (4N). Proliferative inhibition is therefore not a result of entering a quiescent G0 phase,

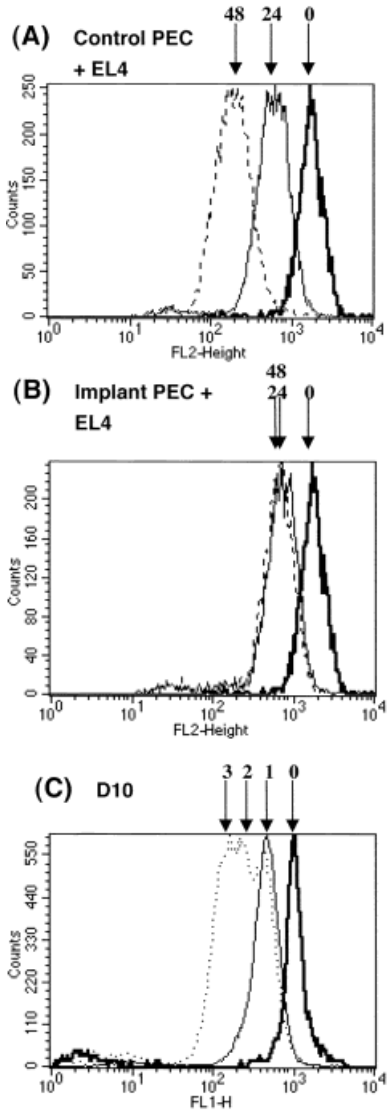

Fig. 5. Proliferation is blocked after one cell division. EL4 cells stained with PKH26 were co-cultured with control PEC (A) or implant PEC (B) for 24 and 48 h before harvest and analysis by FACS. With each cell division fluorescence intensity halves. The arrow marked 0 indicates the original intensity of cells that were fixed immediately after staining. The arrows marked 24 and 48 indicate fluorescence intensity of cells harvested after 24 or 48 h culture with PEC. This experiment was repeated with CFSE staining with identical results. (C) D10.G4 cells stained with CFSE were stimulated (50 μg/ml conalbumin) with implant PEC (thin line) or control PEC (broken line) for 3 days. The marked arrows indicate the number of cell divisions and the thick line represents the original intensity of the D10 cells. This result is representative of two experiments.

but might instead be associated with accumulation of cell cycle proteins.

3 Discussion

Understanding the mechanisms that inhibit or regulate cellular proliferation is of general interest to immunologists and cell biologists, as well as tumor biologists. We have previously found that filarial nematodes induce the recruitment and/or activation of a suppressor cell type in the peritoneal cavity of mice, which profoundly inhibits the proliferation of a range of murine cells. The recruitment of these anti-proliferative cells is induced by the excretory/secretory products of the worms [21], and is dependent on IL-4 production by the host [16]. In this study, we have identified this suppressive cell type as an IL-4 dependent F4/80$^+$ macrophage. We also found that the anti-proliferative effect is mediated by cell-to-cell contact, occurring even after fixation of the suppressive cells. Most surprisingly, the suppressive ability of the parasite-recruited macrophages could transcend the species barrier, significantly reducing the proliferation of a wide range of human tumor cell lines. These data suggest that filarial nematodes can induce the activation of a macrophage population that inhibits proliferation of cells in the local vicinity via a novel receptor-mediated mechanism.

We have yet to identify the exact nature of the cell surface molecule(s) involved in inhibiting proliferation. The observation that the suppressive macrophages affect such a wide range of human tumor cells, as well transformed mouse cells, strongly suggests the engagement of a highly conserved receptor. Based on our findings, it is tempting to suggest that parasitic nematode infection could have a beneficial effect against tumor expansion as a by-product of recruiting macrophages that suppress proliferation. However, the effect of these cells is probably very limited because of the local nature of receptor-mediated mechanisms. Furthermore, the reversibility of the proliferative block would allow any dividing cells that migrate away from the vicinity of the suppressor cells to resume proliferation. Nonetheless, it would be interesting to investigate if lymphomas are less prevalent in regions endemic for filariasis.

It is unusual for growth regulatory mechanisms to induce a cell cycle block in G2/M as well as G1. The cell cycle machinery usually responds to external stimuli (such as mitogenic or anti-mitogenic factors) only during a defined window of time, from the beginning of G1 to the restriction point (*R*) [22–24]. After *R*, cells are relatively refractory to most external stimuli, the remaining phases of the cell cycle being executed in a quasi-automatic

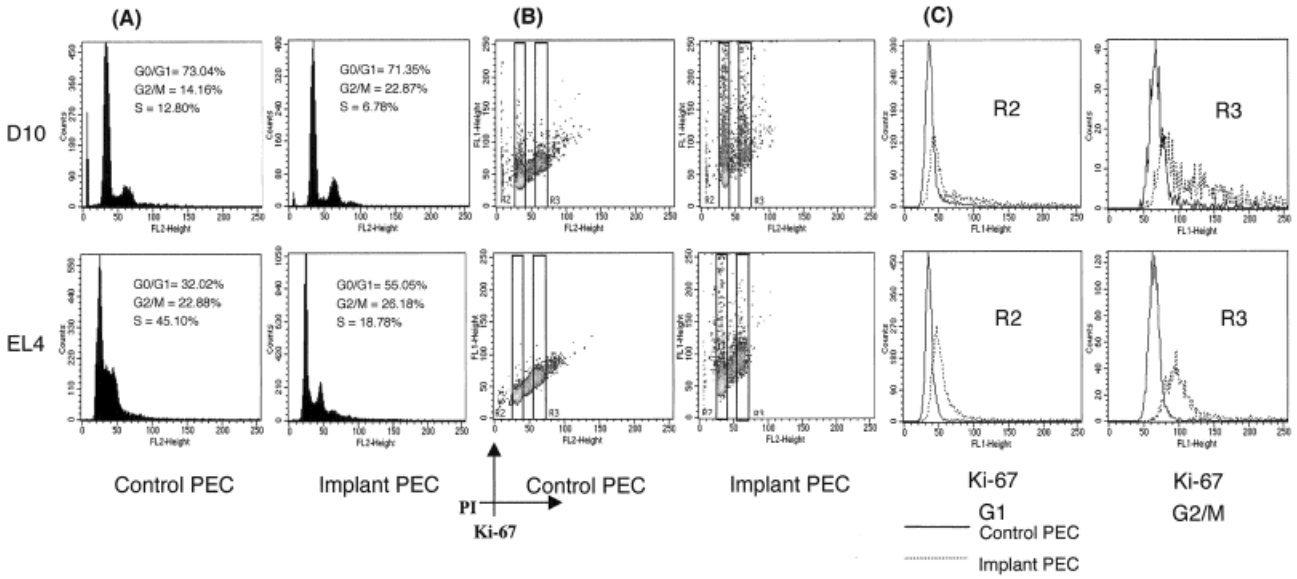

Fig. 6. Cell cycle analysis of PEC-exposed cells. (A) Histograms of D10.G4 and EL4 cells stained with propidium ioidide (PI) 48 h after co-culture with either control or implant PEC. In both cell lines, implant PEC exposed cells have a decreased percentage of cells in S phase, and an accumulation of cells in G1 and G2/M. Results shown are representative of three independent experiments. (B) FACS analysis of cells double stained with Ki-67 as well as PI after 48 h co-culture with PEC. Ki-67 expression is shown on the Y-axis (FL-1) and PI staining is shown on X-axis (FL-2). The R2 gate represents 2N cells in G1. The R3 gate represents 4N cells in G2/M. (C) Histogram analysis of Ki-67 expression in G1 (R2) and G2/M (R3) cells, using gates shown in B. Implant PEC exposed cells (dotted lines) have increased expression of Ki-67 in both these phases of the cell cycle, compared to control (unbroken line). Results shown are representative of three independent experiments.

fashion [24]. Thus, the majority of anti-proliferative signals would lead to a G1 arrest of the cell cycle, or entry into the quiescent G0 state. A G2/M arrest usually occurs only as a result of interference with DNA replication [25, 26]. The effect on two phases of the cell cycle raises the possibility that more than one mechanism could be acting in concert to inhibit proliferation. Alternatively, the suppressive macrophages may be slowing down the cell cycle by delaying the passage through the checkpoint controls at G1 and G2/M, while having no effect on progression through S phase.

It is particularly interesting that the accumulation of cells at G1 and G2/M is associated with an increased level of Ki67 protein. Although the mAb Ki-67 has been used to identify proliferating cells since 1983 [27], the function of the protein recognized by this mAb is still uncertain. Antisense oligonucleotide treatment or microinjection of antibodies into the nucleus will delay cell cycle progression, suggesting that Ki-67 antigen is essential in this context [28, 29]. However, there are no reports linking increased expression with cell cycle arrest. Interestingly, one of the few factors which can lead to a combined G2/M and G1 block is inhibition of the proteasome [30], which leads to an accumulation of ubiquitinated proteins, including the various cyclins necessary for cell cycle progression [30, 31]. Perhaps the anti-proliferative macrophages engage receptors that lead to changes in protein degradation of the suppressed cells, leading to accumulation of cell cycle proteins such as Ki-67. We plan to establish whether Ki-67 accumulation in G1 and G2/M cells is directly involved in the mechanism of suppression or merely an indirect consequence of cell cycle arrest. We are also investigating the effect of suppression on the levels of cyclins in the non-proliferating cells.

Protozoan parasites (*Trypanosoma brucei* and *Toxoplasma gondii*) induce IFN-γ-dependent macrophages that suppress splenocyte proliferation via NO [8–10]. Unlike these classically activated macrophages, suppressive macrophages that do not rely on IFN-γ for activation have been described as “alternatively activated” (reviewed recently by Goerdt and Orfanos [14]). Alternative activation is associated with type 2 immune response mediators such as IL-4, glucocorticoids, IL-13 and TGF-β. Thus, it is appropriate to categorize the macrophages that are recruited by the nematode *B. malayi* as “alternatively activated”. Further evidence for the activation state of these macrophages comes from our recent analysis of gene expression. A small EST dataset has

revealed that the suppressive macrophages highly express many genes associated with alternative activation and induced by type 2 cytokines (unpublished observation). These include type I arginase [32, 33] and the chemokine C10 [34].

More interestingly, we have found that these macrophages do not exert their suppressive effects via well-known mediators such as PGE_2, IL-10 and TGF-β [15, 35] but instead act through direct cellular contact. Whether suppressive macrophages are recruited by the parasite during a natural filarial infection to modulate the immune response, or they perform some role in preventing immunopathology, remains to be established. The possibility that these cells act to induce regulatory T cells in response to infection is another exciting prospect that we are currently testing. Recently, we have found that these suppressive macrophages can drive Th2 differentiation when used as APC to prime naive T cells from TCR-transgenic mice [35]. The parasite-induced macrophages prevent Th1 differentiation by the inhibition of early IFN-γ production via TGF-β. Interestingly, while TGF-β is involved in Th2 differentiation, it is not involved in the proliferative suppression [15, 35]. The biased induction of Th2 cells by these alternatively activated macrophages, as well as their anti-proliferative effects, is consistent with their potential role as important anti-inflammatory cells.

In conclusion, we report the observation of a contact-dependent mechanism of inhibiting proliferation that has profound effects on the G1 and G2/M phases of the cell cycle. This novel mechanism of suppression by filaria-induced macrophages (which is dependent on host IL-4) can also significantly reduce the proliferation of a wide range of human tumor cells. Thus, the identification of the molecules involved may provide new insight into tumor biology as well immunology and parasitology.

4 Materials and methods

4.1 Mouse infection model

Apart from experiments with genetically deficient mice, 6- to 8-week-old CBA/Ca males were used. C57BL/6 IL-4 deficient (IL-$4^{-/-}$) breeding pairs were purchased from B&K Universal Ltd. (North Humberside, GB) with permission of the Institute of Genetics, University of Cologne. C57BL/6 IL-5-deficient (IL-$5^{-/-}$) mice [19] were the kind gift of Prof. Manfred Kopf (Basel Switzerland). *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 μg/ml gentamicin (RPMI wash). Mice were surgically implanted i. p. with six live adult *B. malayi* females. After 3–6 weeks, mice were killed by cardiac puncture and PEC were harvested by thorough washing of the peritoneal cavity with 15 ml RPMI wash (implant PEC). Replicate assays were performed with PEC from individual mice in each experiment, except where cells were pooled due to insufficient cell numbers from single mice (indicated in results). Resident peritoneal cells were harvested in the same way from control non-implanted mice and termed "control PEC".

4.2 Cultures and cell lines

All *in vitro* cultures were carried out in RPMI 1640 (GIBCO) supplemented with 2 mM glutamine, 0.25 U/ml penicillin, 100 μg/ml streptomycin, 5 μM 2-ME and 10 % FCS (complete medium). The Th2 cell clone D10.G4 [36] was maintained in culture as previously described [15]. The murine T cell lymphoma EL4 and the B cell hybridoma HB32 were maintained in complete medium. The tumor cell lines used were acclimatized to RPMI media for at least 2 weeks prior to exposure to PEC. IL-4 production by D10.G4 cells was measured with the NK cell line as previously described [15]. Cell lines used are available from ATCC Rockville, MD.

4.3 Suppression assays

PEC (10^5/100 μl) were allowed to adhere to flat-bottom 96-well plates for 2–3 h at 37 °C. Nonadherent cells and microfilariae were then removed by washing and the adherent cells co-cultured with 10^5 D10.G4, 5×10^4 EL4 or 5×10^4 HB32 cells to a final volume of 200 μl/well. D10.G4 were cultured with conalbumin at a final concentration of 50 μg/ml. After 48 h at 37 °C, 1 μCi [^{3}H] thymidine was added to each well, and plates harvested for counting 16–18 h later. To measure suppression of human tumor lines, 10^4 cells in 100 μl were added to the adherent PEC. To determine if suppressed cells recover the ability to proliferate, EL4, HB32 and D10.G4 were co-cultured in 24-well plates with PEC for 48 h and non-adherent cells then aspirated and further purified using nylon wool columns. The HB32 and EL4 cells were then re-cultured in 96-well plates at the same starting concentration (5×10^4 cells per well), and proliferation assayed by [^{3}H] thymidine incorporation 48 h later. Nylon-wool purified D10.G4 cells (1×10^5 per well) were restimulated with 5×10^5 syngeneic irradiated splenocytes and 50 μg/ml conalbumin in each well, and proliferation was also assayed 48 h later. For investigation into the effect of suppressive PEC on apoptosis and the cell cycle (see Sect. 4.5 below), 24-well plates were used at the cell concentrations described above.

4.4 Analysis of cell contact

Twenty-four-well (0.4 μm pore) transwell cell culture chambers (Costar, GB) were used to separate PEC from

responder cells. Results were identical regardless of whether PEC were cultured in the upper or lower compartments. In each well, 1×10^6 PEC were separated from either 5×10^5 D10.G4 cells and 2.5×10^5 syngeneic irradiated splenocytes and 50 μg/ml conalbumin, or 6×10^5 HB32 cells. Previous experiments have shown that addition of irradiated syngeneic splenocytes does not reverse suppression of D10.G4 cells [15]. The same numbers of cells were cultured together in 24-well plates without a separation membrane as the control. Cultures were incubated for 24 h and pulsed with 50 μCi [^{3}H] thymidine per well. Responder cells were transferred to 96-well plates to be harvested and counted. Fixation experiments were performed by standard methods [37]. Briefly, PEC were incubated in 96-well plates (at the cell numbers described above) with 1 % paraformaldehyde for 15 min, and then lysine wash solution for 30 min, followed by four washes with incomplete RPMI, before being cultured with EL4 and HB32 cells as described above. PEC were also pulsed with 50 μg/ml conalbumin prior to fixation, before being used to stimulate/suppress D10.G4 cells.

4.5 Cell cycle and apoptosis analysis

To investigate changes in the cell cycle as a result of suppression, EL4 and D10.G4 cells were stained with propidium iodide according to the protocol of Darynkiewicz [38]. Briefly, after 24 or 48 h culture with PEC, suppressed cells were fixed with 0.5 % formaldehyde in HBSS, permeabilized with 0.1 % Triton X-100 and 1 % BSA in HBSS, and resuspended in HBSS solution containing 5 μg/ml propidium iodide and 100 units of RNAse A. The cells were then analyzed by a FACScan (Becton Dickinson). Data were analyzed using the cell cycle analysis software Modfit (Verity Software House), which calculates the percentage of cells in the different phases of the cell cycle using a Gaussian model for G0/G1 and G2/M cells, and estimates S-phase cells as a trapezoid component. Suppressed cells were also double stained by incubating with FITC-conjugated Ki-67 (Boehringer Mannheim) prior to incubation with propidium iodide-containing HBSS solution [38]. Cell division of suppressed EL4 cells was investigated with the proliferation dyes PKH26 (Sigma) and CFSE (Molecular Probes) with identical results. PKH26 staining was carried out according to the manufacturer's instructions (Sigma). A modification of Lyons and Parish's technique for CFSE staining was used [20]. Briefly, 1×10^7 cells/ml were incubated with 10 μM CFSE in PBS for 8 min at room temperature. Staining was stopped with an equal volume of FCS, and the cells washed three times with complete medium. PKH26- and CFSE-labeled cells were then co-cultured with control and implanted PEC as described above. A TUNEL kit (Boehringer Mannheim) was used to measure apoptosis after 48 h co-culture with PEC according to the manufacturer's instructions.

4.6 Characterization of suppressive macrophages

Cytocentrifuge preparations were made using a Shandon Cytospin (Shandon, PA). Cells (1×10^5 in 100 μl complete RPMI) were combined with 100 μl FCS. Cytospins were air dried, fixed in methanol and stained with Diff-Quik (Dade, Germany). Photomicrographs and morphological examination were done using a Nikon Microphot-FX microscope. Before magnetic bead cell purification, PEC were passed through a 70-μm cell strainer and purified by centrifugation over Histopaque (Sigma) in order to remove any microfilariae. PEC were then sorted with MS+ or VS+ columns according to the manufacturer's instructions (Miltenyi Biotec). CD11c-positive and -negative cells were purified with FITC-conjugated anti-CD11c (clone HL3, hamster IgG1; Pharmingen) and anti-FITC microbeads. CD11c-negative cells were purified by passing the negative fraction of the first selection column through a second column with a flow resistor. F4/80$^+$ cells were purified with biotin-conjugated F4/80 (rat IgG2b; Caltag) and Streptavidin microbeads. The purity of the sorted cells was checked using a FACScan with CELLQuest software (Becton Dickinson). Positive fractions were typically between 90–95 % pure, and negative fractions were between 98–99 % pure. Purified cells were then co-cultured with D10.G4, EL4 or HB32 cells, as described above.

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