Alternatively Activated Macrophage Possess Antitumor Cytotoxicity That Is Induced by IL-4 and Mediated by Arginase-1

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Summary: Earlier studies have shown that the adoptive transfer of Th2-polarized CD4$^+$ T cells can clear established tumors from mice in an antigen-specific manner. Although eosinophils were implicated in this process, the exact mechanism of tumor clearance and which immune effector cells were involved, remain to be defined. Consequently, experiments were undertaken to elucidate the mechanism of Th2-mediated destruction of B16-F1 melanoma cells by examining the in vitro antitumor activity of leukocytes within a type-2 inflammatory infiltrate. The experimental data show that activation of alternatively activated macrophages (aaMacs) within type-2 infiltrates by IL-4 or IL-13 can inhibit B16-F1 melanoma cell proliferation through a mechanism that is dependent on arginase-1 depletion of L-arginine within the tumor cell microenvironment. Interestingly, whilst at higher E:T ratios aaMac exhibited antitumor activity, at lower E:T ratios aaMacs were observed to enhance rather than inhibit B16-F1 melanoma cell growth. This highlights the fine balance between stimulating the antitumorigenic and protumorigenic properties of aaMacs in tumor immunotherapy protocols.

Key Words: tumor immunity, monocytes/macrophages, cytotoxicity, interleukin-4

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Through understanding and harnessing the antitumor immune response, cancer immunotherapy aims to stimulate the immune system to destroy tumors. Whilst the majority of research into tumor immunotherapy has focused on the tumoricidal activity of CTLs, recently there has been renewed interest in antitumor immune responses mediated by CD4$^+$ T cells. CD4$^+$ T cells have the advantage that, unlike CTLs, they can respond to MHC class I negative tumors as they can recognize tumor-associated antigens presented on MHC class II molecules by bystander antigen presenting cells, a process that is less susceptible to immune evasion by tumor cells.[1–3] In particular, there is emerging evidence that CD4$^+$ T cells expressing a type-2 cytokine profile (CD4$^+$ Th2 cells) can be particularly effective at clearing CTL-resistant tumors.[1–5]

We, and others, have earlier shown that CD4$^+$ Th2 cells are able to induce the clearance of established tumors in mice.[2–5] CD4$^+$ Th2 cell-mediated antitumor immunity seems to be dependent on the production of IL-4,[6–9] and the recruitment into tumors of tumoricidal myeloid cells such as eosinophils, [2–4,7–11] often in collaboration with macrophages.[7,9–11] Whilst eosinophils have been strongly implicated in this process,[2,12] the exact effector cells and cellular mechanisms by which these CD4$^+$ T cells induce tumor clearance remains to be elucidated. In addition to eosinophils, Th2 cells and IL-4 can also induce an influx of macrophages into tumors,[7,9–11] suggesting that macrophages may also play a role in Th2-mediated tumor clearance.

The ability of macrophages to destroy tumor cells in vitro is well documented.[3,13,14] Known mediators of macrophage cytotoxicity include NO,[15–17] TNF-α,[18,19] cytolytic proteases,[18] and the generation of reactive oxygen species such as hydrogen peroxide and superoxide after activation of antibody dependent cellular cytotoxicity.[19–21] Macrophage antitumor cytotoxic activity is traditionally associated with a type-1 immune response. Indeed, with the exception of antibody dependent cellular cytotoxicity, which has been reported to be mediated by Th2 cytokine dependent antibody subclasses,[22] macrophage cytotoxic activity is typically upregulated by the type-1 cytokine IFN-γ. This cytotoxicity is mediated by mechanisms such as NO production and secretion of TNF-α.

Conversely, in response to the type-2 cytokines IL-4 and IL-13, macrophages differentiate into a phenotype known as alternatively activated macrophages (aaMacs). This macrophage subpopulation is characterized by high levels of arginase-1 production,[23] and has recently been shown to play an important role in type-2 immunity associated with the clearance of the gastrointestinal parasite *H. polygyrus* in mice.[24] Here, we describe studies undertaken to examine the in vitro cytotoxic activity of leukocytes within a type-2 inflammatory infiltrate against B16-F1 melanoma cells. The data presented here show that activation of aaMacs present in type-2 inflammatory infiltrates by IL-4 or IL-13 can induce antitumor activity against B16-F1 melanoma cells through a mechanism that is dependent on arginase-1 depletion of L-arginine in the tumor cell microenvironment.

MATERIALS AND METHODS

Mice

C57BL/6 and OT-II male mice were sourced from the Animal Resources Centre (ARC), Perth, Australia and the

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Animal Services Division at the Australian National University (ANU), Canberra, Australia, respectively. All mice were housed in specific pathogen free facilities at the John Curtin School of Medical Research (JCSMR) at ANU. Mice were treated in accordance with ANU and National Health and Medical Research Council animal experimentation guidelines. Mice used in experiments were aged between 6 and 8 weeks.

General Reagents

RPMI-1640, D-MEM, and F15 media were sourced from Gibco BRL (Grand Island, NY) and, along with HBSS, prepared by the JCSMR media facility. The mouse macrophage-specific mAb, F4/80, was purchased from Serotec (Kidlington, UK). mAb to mouse CD11b, CD80, CD86, and IA^b (MHC class II) were purchased from BD Biosciences (San Jose, CA). Recombinant mouse IL-4 and IL-13 were purchased from Peprotech (Rockyhill, NJ). N^G-hydroxy-L-arginine (L-NOHA), OVA and L-arginine were purchased from Sigma Aldrich (St Louis, MO) and S-(2-boronoethyl)-L-cysteine (BEC) from Calbiochem (Darmstadt, Germany). Recombinant arginase-1 was a kind gift of Dr Bill Cowden (JCSMR, ANU).

Th2 Peritoneal Exudate Cells (PEC)

C57BL/6 mice were sensitized by i.p. injection of 50 μg of OVA/10% aluminum hydroxide in 200 μL of 0.15 M NaCl on day 0, followed by i.p. challenge with 50 μg of OVA in 200 μL of 0.15 M NaCl on days 12, 13, and 14, before harvesting PEC on day 15 by washing the cavity 3 times with HBSS (5 mL/wash) and any contaminating red blood cells lysed.

Purification of Peritoneal Macrophages

Macrophages were purified from OVA-induced PEC by sorting on a BD FACS Vantage (BD Bioscience, San Jose, CA) based on forward and side scatter parameters and polarized light. The purity of the macrophage enriched population was more than 95% as determined by differential staining with May-Grünwald Giemsa and expression of the cell surface molecules CD11b, F4/80, CD80, CD86, and MHC class II by flow cytometry. Flow cytometric acquisition was carried out on a FACS LSR I (BD Biosciences, San Jose, CA) and analysis done using FlowJo Software (Tree Star, Ashland, OR).

Tumor Cell Proliferation Assay

B16-F1 melanoma cells were seeded into 96-well plates at 4×10^3 cells/well in F15/10% FCS and grown at 37°C in 5% CO_2 for 20 to 24 hours before coculturing with effector cells at various E:T ratios in RPMI/10% FCS alone or supplemented with cytokines. After 30 hours of coculture 0.5 μCi of [^{3}H]-thymidine was added to each culture well in 20 μL of RPMI/10% FCS and [^{3}H]-thymidine incorporation measured for 18 hours. At the end of the culture period, cell supernatants were removed and the cells washed with 200 μL of PBS, then deadhered using 100 μL/well of trypsin/EDTA at 37°C for 7 minutes. Cell cultures were stored at −20°C until harvesting to measure [^{3}H]-thymidine incorporation in counts per minute (cpm) as earlier described.[12] Results were calculated as either (i) B16-F1 proliferation (% of control) = $(cpm_{co\text{-}culture} - cpm_{effector\ cells\ alone})/cpm_{B16\text{-}F1\ cells\ alone} \times 100$ or (ii) reduction in B16-F1 proliferation (%) = $100 - [(cpm_{co\text{-}culture} - cpm_{effector\ cells\ alone})/cpm_{B16\text{-}F1\ cells\ alone} \times 100]$. In some experiments, the effect of the addition of arginase inhibitors (BEC and L-NOHA) or exogenous L-arginine on tumor cell viability was assessed.

Fluorescence Cytotoxicity Assay

B16-F1 melanoma cells were seeded into 96-well plates at 4×10^3 cells/well in F15/10% FCS and grown at 37°C in 5% CO_2 for 20 to 24 hours before coculturing with sort-purified macrophages at an E:T ratio of 20:1 in the presence of IL-4 (10 ng/mL) for 2 days. Viable cells remaining in culture were then enumerated by flow cytometry, using Flow-Count counting beads and the viability and apoptotic reagents, Hoechst 33258 and Annexin V, with B16-F1 melanoma cells being discriminated from macrophages using a FITC-conjugated anti-CD45.2 mAb (Pharmingen, expressed by macrophages but not by B16-F1 melanoma cells). A total of 4000 counting beads were added to each sample, thus the ratio of added beads to beads recovered could be used to calculate the total number of cells recovered in each sample.

Cytokine Secretion Assay

Cytokine secretion was measured using a cytokine bead array flex kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Cytokines examined were IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ, and TNF. Flow cytometric acquisition was carried out on a FACS LSRII instrument (BD Biosciences, San Jose, CA) and analysis done using FCAP Array Software (Soft Flow from BD Biosciences, San Jose, CA).

Arginase Activity Assay

Arginase activity of cultured macrophages was measured using a modified Schimke micromethod.[25] In brief, 5×10^5 OT-II induced PEC were cultured in DMEM/10% FCS with IL-4 (10 ng/mL) for 24 hours. At the end of the culture period, cells were washed with PBS, and resuspended in 50 μL of PBS/0.1% Triton X-100 (Sigma, St Louis, MO) containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and stirred for 30 minutes at RT. After the cells were lysed, 50 μL of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 was added, and the enzyme activated for 10 minutes at 55°C. Arginase hydrolysis was done in eppendorf tubes containing 25 μL of 5 mM L-arginine (Sigma, St Louis, MO), pH 7.9, a 25 μL aliquot of the prepared cell lysate added and the mixture incubated at 37°C for 60 minutes. The reaction was stopped with the addition of 200 μL of H_2SO_4:H_3PO_4:H_2O (1:3:7). To quantify urea formation 25 μL of 9% α-isonitrosopropiophenone (ISPF; Sigma, St Louis, MO) in 100% ethanol was added to the mixture and heated at 100°C for 45 minutes then cooled for 10 minutes at RT protected from light. The reaction mixture (200 μL) was transferred to a 96-well microplate and urea formation quantified colorimetrically by measuring the absorbance at 540 nm using a Thermomax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Data were analyzed using SoftMax Pro software (Molecular Devices Corporation, Sunnyvale, CA). A standard curve was prepared using increasing amounts of urea (Ajax Finchem, Seven Hills, Australia) ranging from 625 ng to 40 μg.

Statistical Analysis

Statistical significance was measured using the Alternate Welch *t* test (unless otherwise indicated) done using InStat Software (GraphPad Software, San Diego, CA).

RESULTS

IL-4 Induces Th2 PEC to Inhibit B16-F1 Melanoma Cell Growth and Survival

To determine the effector mechanisms involved in Th2-mediated tumor clearance, we monitored in vitro tumor cell proliferation by [^{3}H]-thymidine incorporation. This assay offered the advantage of measuring both the cytotoxic and cytostatic effects of various treatments on B16-F1 melanoma cells. Furthermore, the 48 hours time period of this assay enabled investigation of the long-term effects on tumor cell survival that may be missed using the classical short-term cytotoxicity assay based on radioactive chromium release. To generate a Th2 inflammatory cell population, C57BL/6 mice were sensitized and challenged i.p. with alum-OVA, a known inducer of OVA-specific Th2 inflammation. Leukocytes recruited into the peritoneal cavity were harvested by peritoneal wash and cocultured with B16-F1 melanoma cells in vitro to determine their cytotoxic or cytostatic activity.

Coculture of Th2 PEC with preseeded B16-F1 melanoma cells resulted in significant decreases in B16-F1 melanoma cell proliferation. Thus, at an E:T ratio of 25:1, the PEC displayed intrinsic antitumor activity decreasing B16-F1 melanoma cell proliferation by 32% (Fig. 1), which was increased to 86% at an E:T of 50:1. Interestingly, the addition of 10 ng/mL of exogenous IL-4 significantly enhanced this antitumor activity, the Th2 PEC completely inhibiting all B16-F1 melanoma cell proliferation at E:T ratios of 25:1 and above. Furthermore at an E:T ratio of just 10:1, Th2 PEC were able to inhibit B16-F1 melanoma cell proliferation by 75% when activated with IL-4 (Fig. 1). Although less potent than IL-4 another type-2 cytokine, IL-13, was also able to promote the antitumor activity of the Th2 PEC, resulting in a 36% decrease in B16-F1 melanoma cell proliferation at an E:T ratio of 10:1 and a complete inhibition of proliferation at E:T ratios of 25:1 and above (Fig. 1). Enhancement of antitumor activity by the type-2 cytokines IL-4 and IL-13 seemed to be stimulating the same inhibitory mechanism as there was no additional antiproliferative activity when IL-4 and IL-13 were combined compared with IL-4 alone. Thus IL-4 alone is sufficient to induce maximal antitumor activity (Fig. 1). Neither IL-4 nor IL-13 treatment had any effect on B16-F1 melanoma cell proliferation in the absence of PEC (data not shown). Similar trends in antitumor activity were observed during coculture of a second type of PEC induced by i.p. injection of Th2-polarised OT-II CD4$^+$ T cells (OT-II PEC; Supplementary Fig. S1, Supplemental Digital Content 1, http://links.lww.com/JIT/A46), although their overall potency was less compared with the OVA-induced Th2 PEC (Supplementary Fig. S2A, Supplemental Digital Content 2, http://links.lww.com/JIT/A47).

To understand the mechanism(s) of IL-4 and IL-13-induced antiproliferative activity against the B16-F1 melanoma cells, it was necessary to first determine the identity of the antiproliferative cells within the PEC population. Examination of May-Grünwald Giemsa-stained cytospin preparations of the OVA-induced PEC population (Fig. 2A) revealed that the exudate predominately consisted of cells of the macrophage or monocyte lineage, but also contained lymphocytes, eosinophils, and very few neutrophils (Fig. 2A).

The high percentage of macrophages in the peritoneal exudate suggests a potential role for macrophages in the observed antitumor activity. Indeed, together with eosinophils, infiltrating macrophages have been observed in tumors during Th2-mediated tumor clearance,[7,9–11] suggesting they may play an active role in tumor destruction. To confirm this hypothesis, the different populations of cells were purified from the OVA-induced PEC by flow cytometry (Fig. 2B) and their relative antiproliferative activity against B16-F1 melanoma cells compared. May-Grünwald-stained cytospin preparations were used to confirm the purity of the FACS-purified populations.

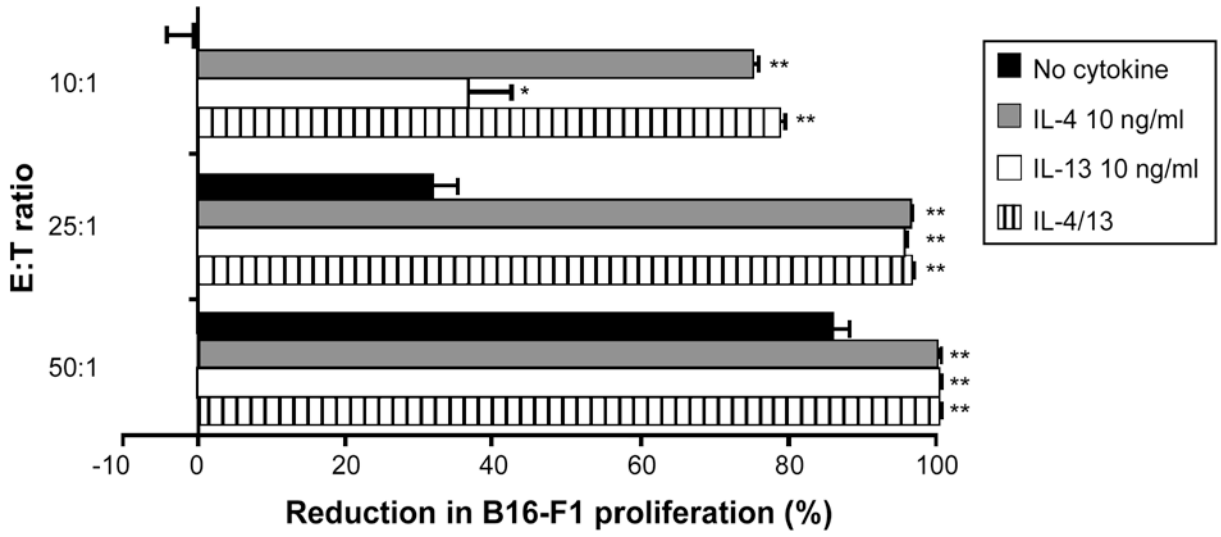

FIGURE 1. Th2 peritoneal exudate cell (PEC) inhibit B16-F1 melanoma cell proliferation. PEC were obtained from C57BL/6 mice sensitized and challenged by i.p. injection of OVA. PEC were cocultured for 48 hours with preseeded B16-F1 melanoma cells with and without cytokines at various E:T ratios. B16-F1 melanoma cell proliferation was quantified by [^{3}H]-thymidine incorporation and the antitumor cell activity expressed as the percentage reduction in B16-F1 cell proliferation. Values represent the mean of triplicate samples ± SEM and are representative of 2 independent experiments. Negative values indicate an increase in B16-F1 melanoma cell growth. *P* values are for the condition shown compared with the no cytokine control at the same E:T ratio. $^{**}P \leq 0.001$.

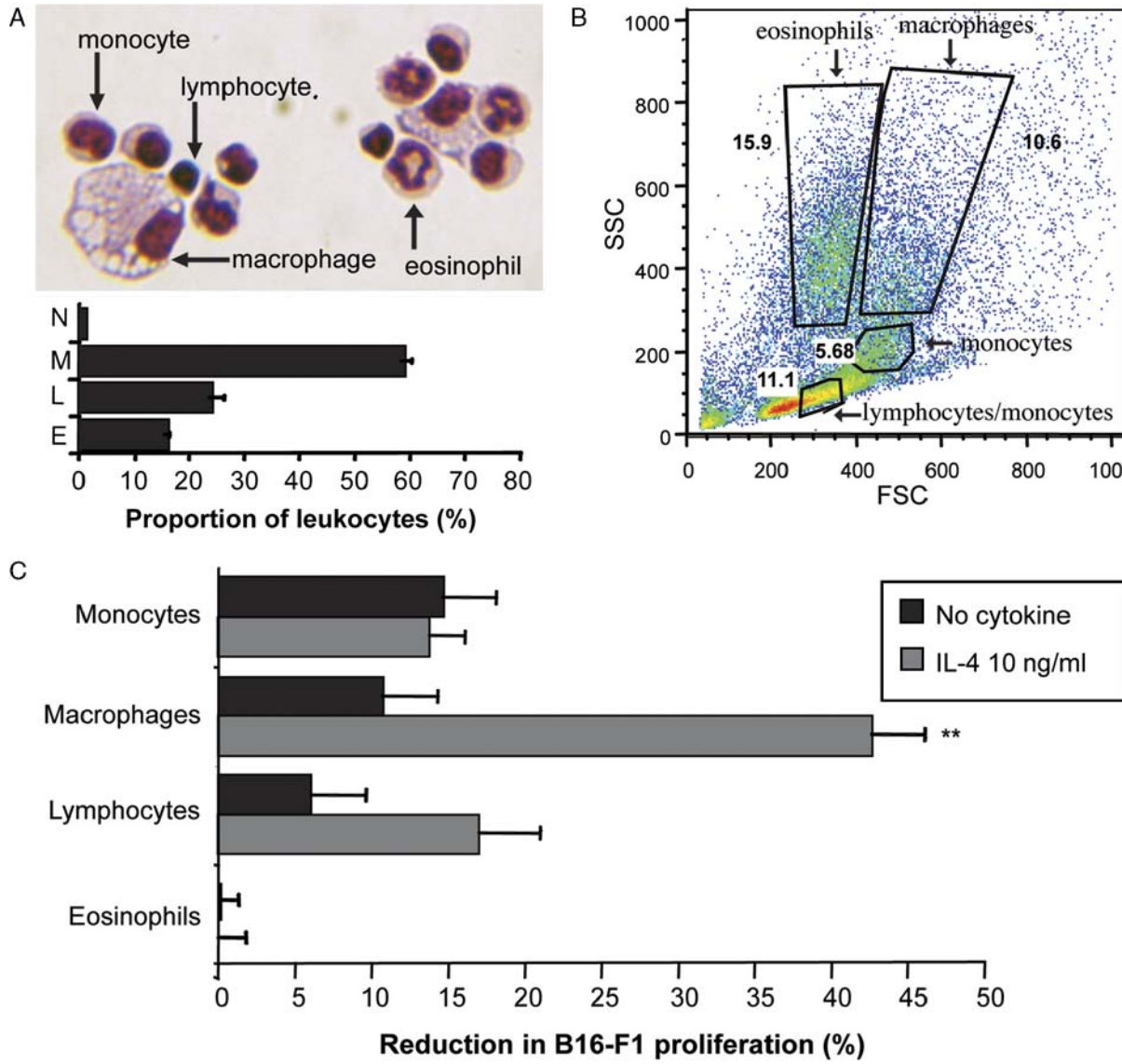

FIGURE 2. B16-F1 melanoma cell proliferation is inhibited by Th2 peritoneal macrophages. A, The percentages of neutrophils (N), macrophages/monocytes (M), lymphocytes (L), and eosinophils (E) within the OVA-induced peritoneal exudate cell (PEC) population were determined from May-Grünwald Giemsa stained cytospin preparations of OVA-induced PEC visualized by bright field microscopy (400 × magnification; 200 cells enumerated/slide). Values are shown as the percentage of total cells and represent the mean of 5 separate experiments ± SEM. B, Macrophage, eosinophil, monocyte, and lymphocyte subpopulations were purified from OVA-induced PEC by flow cytometry based on forward scatter (FSC) and side scatter (SSC) gates shown. C, Purified macrophage, eosinophil, monocyte, and lymphocyte populations were cocultured for 48 hours with preseeded B16-F1 melanoma cells at an E:T ratio of 4:1 with and without cytokines. B16-F1 melanoma cell viability was quantified by [^{3}H]-thymidine incorporation and macrophage antitumor cell activity expressed as the percentage reduction in B16-F1 melanoma cell proliferation. Values represent the mean of triplicate samples ± SEM and are representative of 2 independent experiments. **$P \leq 0.01$ for the IL-4 activated culture compared with the no cytokine control.

At an E:T ratio of 4:1, in the absence of exogenous cytokine, the monocyte, macrophage, and lymphocyte populations all displayed very low levels of antiproliferative activity against the B16-F1 melanoma cells (Fig. 2C). No antiproliferative activity was displayed by the eosinophil population. In contrast, with the addition of exogenous IL-4, only the antiproliferative activity of the macrophage population was significantly increased, resulting in a 43% reduction in B16-F1 melanoma cell proliferation, compared with just 10% for the no cytokine control (Fig. 2C). The level of IL-4-induced antiproliferative activity in the macrophage subpopulation was also significantly ($P < 0.05$) higher than that observed for the other 3 populations. Together these data indicate that it is predominately macrophages within the PEC population that are mediating the IL-4-induced antitumor activity.

The reduction in B16-F1 cell proliferation observed is presumably associated with a reduction in B16-F1 melanoma cell

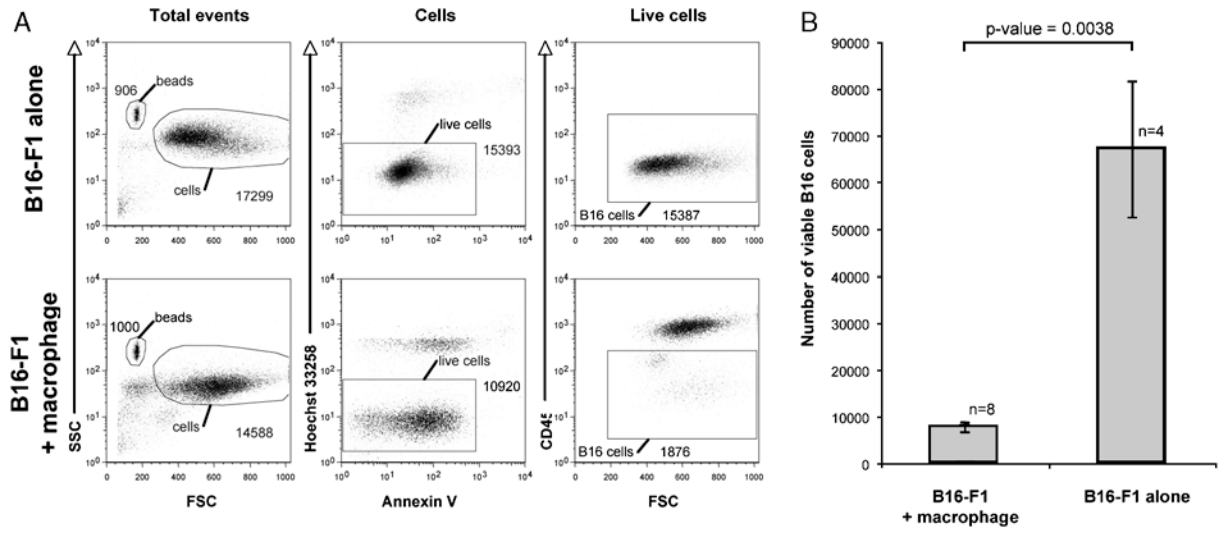

FIGURE 3. Alternatively activated macrophages dramatically inhibit the growth of B16-F1 melanoma cells. Macrophages from the peritoneal cavity of OVA-sensitised mice were cultured with B16-F1 cells at a 20:1 ratio in the presence of IL-4 (10 ng/mL) for 2 days. A, Flow cytometry estimate of viable B16-F1 cells/well after 2 days culture in the absence and presence of macrophage. Panels show the gating strategy used to differentiate between cells and counting beads based on forward and side scatter (first panels), live and dead or apoptotic cells based on Hoechst 33258 and Annexin V staining (second panels) and B16-F1melanoma cells from macrophages based on CD45 expression (third panel). Numbers refer to events recovered in adjacent gates. B, Total number of viable B16-F1 melanoma cells remaining in culture. Total viable B16-F1 melanoma cells recovered from cultures were calculated based on the number of counting beads and viable B16-F1 melanoma cells recovered by flow cytometry. Note: n is the number replicates in each group and the *P*-value was calculated using a 2-tailed, unpaired, unequal variance, *t* test. Data are representative of 3 independent experiments.

numbers in the cultures. This interpretation was validated using a fluorescence-based assay that determined the number of live B16-F1 melanoma cells based on Hoechst 33258 and Annexin V staining (Fig. 3). In the presence of aaMac and IL-4, the number of live B16-F1 melanoma cells was very substantially and significantly reduced compared with B16-F1 melanoma cells cultured alone (Fig. 3B). Thus, the reduction in B16-F1 proliferation seen by [^{3}H]-thymidine incorporation corresponds with a decrease in viable cell numbers, although this assay cannot distinguish between whether the aaMac are directly cytotoxic or cytostatic toward B16-F1 melanoma cells in vitro.

Th2 Peritoneal Macrophages Possess Protumor and Antitumor Activities That Are Moderated by IL-4

Morphologic examination of the flow cytometry purified macrophage population by May-Grünwald Giemsa staining of cytospin preparations, revealed that the purified cells were predominately large activated macrophages (> 90%), not monocytes (< 10%) (Fig. 4A, upper panel). Furthermore, analysis of the expression of cell surface markers by flow cytometry showed that 95% to 100% of the purified cells expressed the macrophage markers CD11b and F4/80 (Fig. 4A, lower panel). In addition, the high level of expression of the costimulatory molecules CD80, CD86, and partially increased levels of MHC class II (Fig. 4A, lower panel) suggest these macrophages are highly-activated mature macrophages. Thus, these macrophages are not myeloid-derived suppressor cells, a type of immature myeloid cell that is $Gr1^+CD11b^+$ but lacks expression of F4/80 and in humans the MHC class II molecule HLA-DR.[26,27]

A titration of E-cell:T-cell ratios revealed that when exposed to IL-4, the purified peritoneal macrophages were effective at inhibiting B16-F1 melanoma cell proliferation at E:T ratios of more than or equal to 4:1 (Fig. 4B). Consistent with earlier data, IL-13 was less effective at inducing macrophage antitumor activity, as a reduction in B16-F1 melanoma cell proliferation was only observed at an E:T ratio of 16:1. At an E:T ratio of 16:1, macrophages also displayed significant intrinsic antiproliferative activity, reducing B16-F1 melanoma cell proliferation by approximately 50%, although macrophages alone were not as effective as macrophages in the presence of exogenous IL-4 or IL-13, with the addition of either cytokine resulting in a more than 95% reduction in B16-F1 melanoma cell proliferation (Fig. 4B). Overall, at all E:T ratios antitumor activity seemed to be not affected or only slightly enhanced by combining IL-4 and IL-13, suggesting that these cytokines may be inducing antitumor cytotoxicity through the same activation pathway. A titration of IL-4 showed that 10 ng/mL was indeed the ideal concentration to induce maximal macrophage cytotoxicity (Fig. 4C).

Interestingly, macrophages also seemed to possess protumorigenic properties at low E:T ratios. In fact at E:T ratios as low as 1:1 or 2:1, macrophages seemed to dramatically increase B16-F1 melanoma cell proliferation compared with the B16-F1 melanoma cells cultured alone (Fig. 4B). This enhanced proliferation was even observed in the presence of IL-4, although to a lesser extent, suggesting that there is a fine balance between macrophage protumorigenic and antitumorigenic properties. A time course analysis showed that the IL-4-induced macrophage antiproliferative activity required 30 hours of coculture for the effect to be observed (Fig. 4D). These data are not consistent with a cytostatic function of aaMac, but suggest

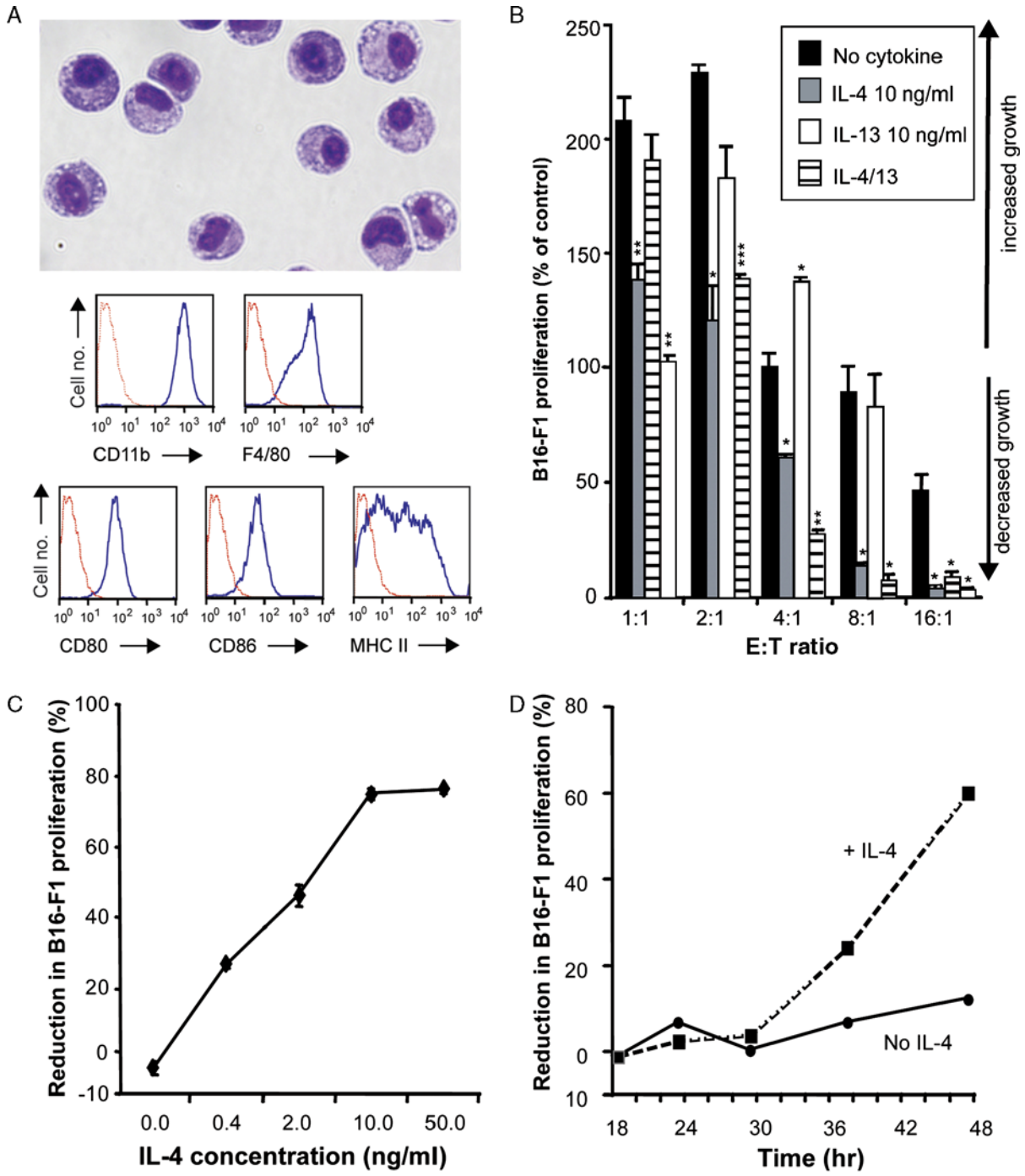

FIGURE 4. Ability of Th2 peritoneal exudate macrophages to modulate B16-F1 melanoma cell proliferation. Peritoneal macrophages were purified from OVA-induced peritoneal exudate cell (PEC) by flow cytometry and cocultured for 48 hours with preseeded B16-F1 melanoma cells to determine their effect on tumor cell proliferation. A, Validation of macrophage purity as revealed by bright field microscopy (400 × magnification) of a May-Grünwald Giemsa stained cytospin preparation (upper panel) and by flow cytometric examination of macrophage-associated cell surface markers (lower panel). Dotted red line in flow cytometry plots represents macrophage autofluorescence and the continuous blue line represents Ab staining. B, Effect of macrophages on tumor cell proliferation at various E:T ratios with and without the cytokines IL-4 and IL-13. C, Macrophage antiproliferative activity at increasing concentrations of IL-4 at an E:T ratio of 4:1. D, A time course of macrophage antiproliferative activity at an E:T ratio of 4:1 with (black squares) or without (black circles) IL-4 (10 ng/mL). B16-F1 melanoma cell proliferation was quantified by [^{3}H]-thymidine incorporation and the effect of Th2 peritoneal macrophages on B16-F1 cell survival expressed as the percentage proliferation (B) and the percentage reduction in B16-F1 cell proliferation (C, D) compared with B16-F1 melanoma cells cultured alone. Values represent the mean of triplicate (B, C) or duplicate (D) samples ± SEM (B, C only). Data are representative of 1 (C, D) or 2 (B) independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ for the cytokine activated culture compared with the no cytokine control.

a delayed cytotoxic effect. This delayed antitumor effect is most likely owing to the time needed for aaMac-derived arginase-1 to deplete L-arginine to levels sufficient to induce tumor cell death (see below). In an attempt to identify other potential molecules involved in modulating B16-F1 melanoma cell proliferation, the cytokine secretion profile of the aaMac population was also examined. It was found that after 48 hours culture the cells secreted, either in the presence of absence of IL-4, modest levels of TNFα (approximately 325-425 pg/mL) but undetectable levels of IFNγ, IL-2, IL-5, and IL-13. TNFα has well established antitumor properties but, in the case of B16 melanoma, only at concentrations more than 1000 pg/mL[28] and, consequently, is unlikely to play any significant role in the antiproliferative activity of aaMac.

IL-4-induced Macrophage Antitumor Activity Is Dependent on Arginase-1

In response to IL-4, macrophages differentiate into alternatively activated macrophages (aaMac) that are characterized by high levels of arginase-1 production.[23] Interestingly, the production of arginase-1 by aaMacs has recently been shown to play an important role in the clearance of the gastrointestinal parasite *H. polygyrus* in mice.[24] Thus it was hypothesized that arginase-1 may also play an important role in IL-4-induced tumor clearance by macrophages, through depleting the essential amino acid L-arginine from the tumor microenvironment.

Initial studies showed that B16-F1 melanoma cells were sensitive to the local depletion of L-arginine as the addition of recombinant arginase-1 at either 0.5 or 1.0 U/mL to the cell cultures resulted in an almost 100% reduction in B16-F1 melanoma cell proliferation (Fig. 5A). These data suggested that aaMac derived arginase-1 would be an effective way of inhibiting B16-F1 melanoma cell growth and, therefore, the effects of the arginase-1 inhibitors N^G-hydroxy-L-arginine (L-NOHA) and S-(2-boronoethyl)-L-cysteine (BEC) on the antiproliferative activity of peritoneal macrophages was investigated. Indeed, addition of L-NOHA (500 μM) or BEC (250 μM) at inhibitor concentrations shown to block arginase-1 activity (Supplementary Fig. S3, Supplemental Digital Content 3, http://links.lww.com/JIT/A48) resulted in inhibition of IL-4-induced macrophage antiproliferative activity by 100% and 80%, respectively (Fig. 5B). Furthermore, the macrophage antiproliferative activity against B16-F1 melanoma cells was abrogated by increasing the concentration of L-arginine within the culture medium, thereby blocking the action of arginase-1 (Fig. 5C). For example, the initial L-arginine concentration in the culture medium was 200 μg/mL, and simply doubling this concentration to 400 μg/mL, reduced macrophage antiproliferative activity by 60%, whilst increasing the L-arginine concentration 5-fold to 1 mg/mL almost completely inhibited all antitumor activity (Fig. 5C). Interestingly, the intrinsic antitumor activity of aaMac evident at a higher E:T ratio (8:1) could also be blocked by the addition of L-NOHA (Fig. 5B), confirming that this activity is also working through arginine-1 production and, importantly, that arginase-1 mediated aaMac antitumor activity can occur without the addition of exogenous IL-4.

DISCUSSION

The ability of macrophages to destroy tumor cells in vitro is well documented.[3,13,14] However, macrophage antitumor cytotoxic activity is traditionally associated with classically-activated macrophages that are generated by a type-1 immune response and whose antitumor activity is typically upregulated by the type-1 cytokine IFN-γ. The experimental data presented in this paper show for the first time the ability of aaMacs to kill tumor cells in vitro, a process that could be enhanced by activation with exogenous IL-4 or IL-13 (Fig. 2D, Fig. 3B and Fig. 4). The antitumor activity seems to be mediated through depletion of L-arginine by arginase-1 within the tumor microenvironment, as shown by a reduction in the antitumor activity of the aaMacs treated with the arginase-1 inhibitors L-NOHA and BEC (Fig. 4B) or excess L-arginine (Fig. 5C). In fact, aaMac are characterized by high levels of arginase-1 that degrades L-arginine to produce L-ornithine and urea. Furthermore, the sensitivity of many tumors to L-arginine depletion is well-documented,[29–32] with data presented here showing that the B16-F1 melanoma cells are also highly sensitive to L-arginine depletion by arginase-1 (Fig. 5A). Thus, an arginase 1-dependent cytotoxic mechanism for aaMac is consistent with these 3 observations. Indeed, LPS and zymosan-activated macrophages have earlier been shown to kill mouse lymphoma cells and rat sarcoma cells in vitro through arginase-depletion of L-arginine, with the addition of extra L-arginine inhibiting macrophage cytotoxic activity when added in the first 18 hours of culture.[33] Interestingly, this latter observation suggests that at least 18 hours is required to deplete the L-arginine from the culture media and begin to induce cell damage, which is consistent with the data presented here showing that a decrease in B16-F1 melanoma cell proliferation was only observed after more than 30 hours of coculture (Fig. 4D).

Although the antitumor cytotoxic activity of macrophages is well established in in vitro studies, controversy surrounds the role of macrophages in tumor progression in vivo. The tumor microenvironment is believed to suppress the cytotoxic activity of macrophages, as tumor-associated macrophages (TAMs) have been shown to exhibit a greatly reduced ability to lyse tumor cells. Furthermore, TAMS are less able to present tumor-associated antigens to T cells, and display reduced expression of immunostimulatory cytokines that are important in stimulating antitumor cytotoxic activity in CTLs and NK cells.[34] In fact, TAMs are considered to have a negative effect on antitumor immunity as they are believed to suppress immunosurveillance by inhibiting CTL activation, proliferation, and therefore, CTL-mediated cytotoxicity,[35–38] and promoting tumor growth,[34,39–42] angiogenesis, and metastasis.[34,43–45] Consistently, many clinical studies have correlated a high number of TAMs with a poor patient prognosis.[34,45]

It is therefore, interesting to note that the studies reported here show that at low E:T ratios (ie, $< 4:1$), macrophages actually increased tumor growth, even after stimuli that resulted in substantial inhibition of tumor growth at higher E:T ratios ($\geq 4:1$). However, as macrophage antitumor activity seems to be mediated through the depletion of L-arginine by aaMac-associated arginase-1, this finding is not surprising, as a large amount of arginase-1 may be required to degrade the high level of L-arginine in the culture medium. In contrast, at lower E:T ratios, L-arginine depletion would be insufficient to have a negative effect on tumor growth and instead, the growth promoting properties of the macrophages becomes dominant. A number of studies have shown that TAMs can

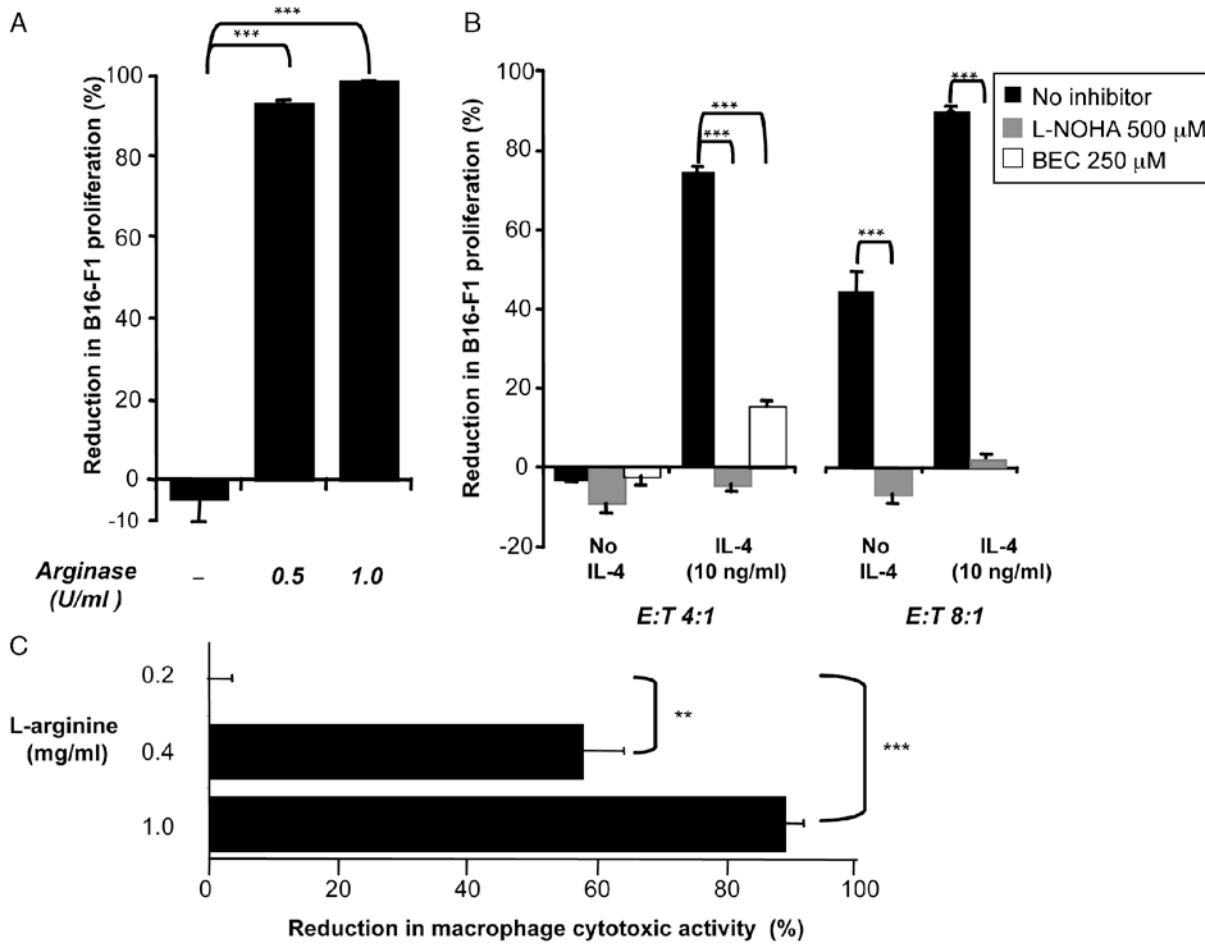

FIGURE 5. Arginase inhibition abrogates IL-4 induced antitumor activity of Th2 peritoneal exudate macrophages. A, B16-F1 melanoma cells were cultured with recombinant arginase-1 for 48 hours to determine their sensitivity to arginase-depletion of L-arginine. The effect on B16-F1 melanoma cell proliferation was quantified by [^{3}H]-thymidine incorporation. B, Flow cytometry purified macrophages were cocultured for 48 hours with preseeded B16-F1 melanoma cells at E:T ratios of 4:1 and 8:1 with or without IL-4. The arginase inhibitors N^G-hydroxy-L-arginine (L-NOHA) and S-(2-boronoethyl)-L-cysteine (BEC) were used to assess the effect of arginase inhibition on antitumor activity. B16-F1 melanoma cell viability was quantified by [^{3}H]-thymidine incorporation and macrophage antitumor activity expressed as the percentage reduction in B16-F1 melanoma cells proliferation. Values represent the mean of triplicate samples ± SEM. Negative values indicate an increase in B16-F1 cell growth. C, Different concentrations of exogenous L-arginine were added to assess the effect on macrophage antitumor activity toward B16-F1 melanoma cells. Results show the percentage reduction in macrophage antitumor activity. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Data are representative of 1 (A), 2 (C), and 3 (B) independent experiments.

express factors that may stimulate tumor cell proliferation and survival, such as epidermal growth factor,[39,40] platelet-derived growth factor, TGF-β, hepatocyte growth factor, and FGF-2.[34] In fact, aaMacs have been shown to secrete TGF-β,[23] however, further work would be required to determine the factors driving enhanced B16 melanoma proliferation observed in our model. Thus, the relationship between macrophages and tumor growth can be likened to a situation in which at very low number of TAMs, there are insufficient levels of mediators to either enhance or inhibit tumor growth (Fig. 6). As the number of TAMs/aaMac increases the production of growth factors and proangiogenic molecules promotes tumor growth. However, above a certain E:T ratio, particularly in a type-2 inflammatory environment, the number of aaMac becomes sufficient to release enough arginase-1 to deplete L-arginine from the tumor microenvironment and inhibit tumor growth (Fig. 6). For the in vitro studies reported here, this critical E:T ratio was 4:1, although it is likely that a much lower E:T ratio of macrophages to tumor cells may be required in vivo in which the local concentrations of L-arginine would be expected to be substantially lower than in tissue culture media. For example, the level of L-arginine in RPMI-1640 medium (950 μM) is more than 20 times higher than the reported level of L-arginine in mouse plasma (44 μM).[46]

At first glance this finding seems to be at odds with clinical studies suggesting that the higher the number of TAMs, the poorer the patient prognosis.[45] However, it is unclear whether these TAMs are of the aaMac phenotype, which is essential for tumor clearance based on L-arginine depletion. This opens the door for potential immunotherapies aimed at recruiting large numbers of aaMacs into tumors or "re-programming" TAMs toward an aaMac phenotype, possibly through IL-4-mediated therapy that

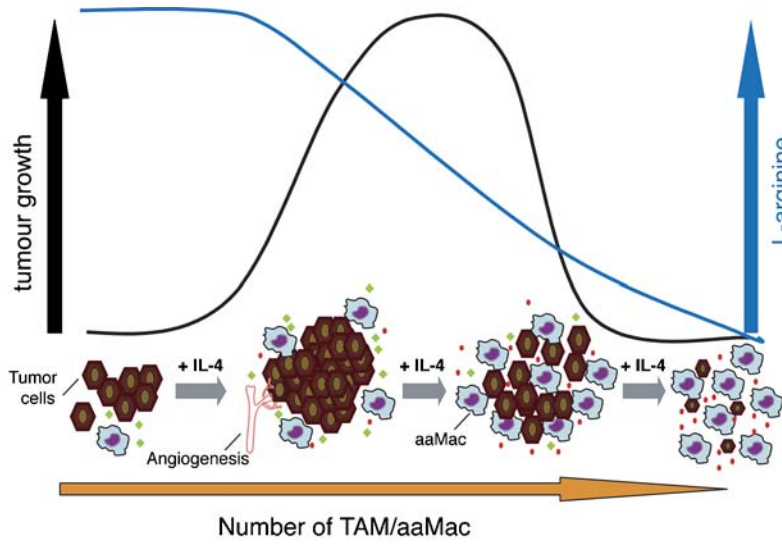

FIGURE 6. Schematic model of the relationship between the number of tumor-associated macrophages (TAMs) and tumor growth. TAMs produce growth factors (green diamonds) that may promote tumor growth (black line) and angiogenesis. In response to type-2 cytokines TAMs become alternatively activated macrophages (aaMac) and produce arginase-1 (red dots) that depletes L-arginine (blue line) from the tumor microenvironment, thereby inhibiting tumor growth. However, the number of TAMs (and aaMac) must reach a critical level before enough arginase-1 is produced to effectively deplete the L-arginine and outweigh the protumorigenic properties of the TAM-derived growth factors.

may then induce tumor clearance. Interestingly, it has recently been suggested that the reprogramming of macrophages by IL-12 can reactivate NO-mediated cytotoxic activity against mouse and human tumor cells in vivo,[47,48] suggesting that the same approach could be used with IL-4 activation of arginase-1. In contrast to our observations, several studies have suggested that tumor-associated aaMacs may inhibit CTL-mediated antitumor responses and therefore, be detrimental to tumor clearance.[37,38] In fact, in 1 study suppression of CTL activity was linked to arginase-1 production,[37] with the depletion of L-arginine inhibiting reexpression of CD3 and antigen-specific activation of CTLs. Whilst these findings must of course be considered in any attempts to generate aaMac-mediated tumor immunotherapies, any inhibition of CTL activity is unlikely to have significant impact on tumors that are themselves sensitive to L-arginine depletion and/or are CTL-resistant as is the case for the B16-F1 melanoma used in the study we have presented here.

In recent years, the discovery of the protumor properties of TAMs has complicated the earlier belief that macrophages could be used in immunotherapy to control tumor growth. However, the experimental results presented here show that in addition to caMacs, IL-4-activated aaMacs possess antitumor cytotoxic activity against tumors. Furthermore, it is clear that when present in low numbers aaMacs may also possess protumorigenic properties that must be balanced with the antitumor effects of aaMacs to develop effective cancer immunotherapies.

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