Reactive Oxygen Species and 12/15-Lipoxygenase Contribute to the Antiproliferative Capacity of Alternatively Activated Myeloid Cells Elicited during Helminth Infection[1]

Lea Brys,* Alain Beschin,[2]* Geert Raes,* Gholamreza Hassanzadeh Ghassabeh,* Wim Noël,* Jef Brandt,† Frank Brombacher,‡ and Patrick De Baetselier*

Understanding the role of CD11b$^+$GR-1$^+$ myeloid suppressor cells in the immune suppression and immunoregulation associated with a variety of diseases may provide therapeutic opportunities. In this article, we show, in a model of helminth infection, that CD11b$^+$GR-1$^+$ myeloid suppressor cells but not CD11b$^+$F4/80^{high} mature macrophages expanded in the peritoneal cavity of BALB/c mice implanted with *Taenia crassiceps*. Peritoneal cell populations from early stage-infected animals impaired T cell proliferation by secreting NO. Yet, they lost their ability to secrete NO in the late stage of infection. Concomitantly, their capacity to exert arginase activity and to express mRNAs coding for FIZZ1 (found in inflammatory zone 1), Ym, and macrophage galactose-type C-type lectin increased. Furthermore, cells from early stage-infected mice triggered T cells to secrete IFN-γ and IL-4, whereas in the late stage of infection, they only induced IL-4 production. These data suggest that CD11b$^+$GR-1$^+$ myeloid suppressor cells displaying an alternative activation phenotype emerged gradually as *T. crassiceps* infection progressed. Corroborating the alternative activation status in the late stage of infection, the suppressive activity relied on arginase activity, which facilitated the production of reactive oxygen species including H_2O_2 and superoxide. We also document that the suppressive activity of alternative myeloid suppressor cells depended on 12/15-lipoxygenase activation generating lipid mediators, which triggered peroxisome proliferator-activated receptor-γ. IL-4 and IL-13 signaling contributed to the expansion of myeloid suppressor cells in the peritoneal cavity of *T. crassiceps*-infected animals and to their antiproliferative activity by allowing arginase and 12/15-lipoxygenase gene expression. ***The Journal of Immunology,*** **2005, 174: 6095–6104.**

About 3 decades ago, non-B, non-T accessory cells hampering immune responses without prior activation were described as natural suppressor cells (1, 2). Recent evidence suggests that these cells are related closely to a population of suppressive cells that express surface markers common to the macrophage (CD11b) and the granulocyte (GR-1) lineage (3, 4).

Myeloid suppressor CD11b$^+$GR-1$^+$ cells represent an intrinsic part of myeloid cell differentiation in the bone marrow (5). They are present in healthy individuals, where their main function in vivo is probably to restrain overwhelming immune responses. However, myeloid suppressor cells (MSC)[3] accumulate in a variety of immune conditions associated with depression of adaptive immune responses. As such, they may constitute a barrier to the therapy of various diseases. Conversely, they may represent therapeutic opportunities to moderate unwanted immune responses, occurring, for instance, during autoimmune diseases or allograft rejection. Consequently, the mechanisms by which MSC influence immune responses were addressed critically in the last years.

MSC interfere with the activation and proliferation of B cells and CD8$^+$ and CD4$^+$ T cells. They can also cause cell death by apoptosis (4, 6). Two major populations of MSC were characterized, based on their differential L-arginine metabolism and the cytokine environment in which they develop. Classical MSC, elicited by IFN-γ released by activated Th1 cells, metabolize L-arginine via inducible NO synthase (iNOS), producing NO and L-citrulline. They were described in systems that include strong immunization procedures (7–10), chemotherapy with cyclophosphamide (3, 11), tumor growth (3, 11–16), as well as during bacterial (17, 18) and American trypanosome (19) infections. Classical MSC inhibit T cell proliferation via their NO secretion, which is elicited by IFN-γ and a yet unknown contact between MSC and T cells. Alternative MSC act on L-arginine via arginase activity to produce urea and L-ornithine. To date, they were found in tumor-bearing mice, being elicited by IL-4 or IL-13 released by Th2 cells or CD1d-restricted NKT cells (20–23). Their suppressive activity depends on a cell

*Department of Cellular and Molecular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Brussels, Belgium; †Diergeneeskunde, Instituut voor Tropische Geneeskunde, Antwerpen, Belgium; and ‡Health Sciences Faculty, University of Cape Town, Cape Town, South Africa

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[2] Address correspondence and reprint requests to Dr. Alain Beschin, Department of Cellular and Molecular Interactions, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium. E-mail address: abeschin@vub.ac.be

[3] Abbreviations used in this paper: MSC, myeloid suppressor cell; iNOS, inducible NO synthase; ROS, reactive oxygen species; 12/15-LOX, 12/15-lipoxygenase; FIZZ1, found in inflammatory zone 1; L-NMMA, N^G-monomethyl L-arginine; nor-NOHA, *N*(omega)-hydroxy-nor-L-arginine; SOD, superoxide dismutase; NDGA, nordihydroguaiaretic acid; [^{3}H]Thy, [*methyl*-^{3}H]-thymidine; MGL, macrophage galactose-type C-type lectin; KO, knockout; PPAR-γ, peroxisome proliferator-activated receptor-γ; COX, cyclooxygenase.

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contact with T cells, possibly via adhesion molecules, and results from the depletion of L-arginine through arginase activity, hereby favoring the iNOS reductase activity leading to increased production of reactive oxygen species (ROS).

In models where the nematode *Brugia malayi* or the cestode *Taenia crassiceps* is implanted in the peritoneal cavity of mice, infection is characterized by a bias toward a Th2 immune response, alternative activation of myeloid cells, and impaired proliferation of immune cells. However, the nature and mechanisms of action of suppressive cells remain poorly characterized. Indeed, although the suppressive activity was assigned to F4/80$^+$ macrophage-like cells in *T. crassiceps*-infected mice, both F4/80$^-$ and F4/80$^+$ suppressive cells were postulated to be induced during *B. malayi* infection (24–28). In addition, NO-independent suppressive mechanisms were evidenced in the late stage of infection with helminths (24–29), but whether this is valid throughout the infection period is not documented.

In this article, we attempted to get better insight in the phenotype as well as the mechanisms underlying the antiproliferative potential of cells elicited in the peritoneal cavity of BALB/c mice implanted with *T. crassiceps* at different time points postinfection. We report the gradual emergence in the course of infection of alternative CD11b$^+$GR-1$^+$ MSC, out of a population that in the early stage of infection displays the features of classical and alternative activation. The change in the activation status as the infection progresses is paralleled by a switch from NO-dependent to ROS- and/or 12/15-lipoxygenase (12/15-LOX)-dependent suppressive activity. Finally, our data also reveal that the development of this type of MSC depends on IL-4 and/or IL-13.

Materials and Methods

Infection

Female wild-type (Harlan), IL-4$^{-/-}$ (30), or IL-4R$\alpha^{-/-}$ (31) BALB/c were inoculated i.p. with 10 nonbudding Toi strain *T. crassiceps* metacestodes as described previously (27) so that, at the time of the experiment, age-matched animals from both the early (<4 wk) and late (>6 wk) stage of infection were available.

Isolation of peritoneal cell populations

Resident peritoneal cells were collected in 0.34 M sucrose and washed in complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.1 mM nonessential amino acids; all from Invitrogen Life Technologies). Adherent cells were recovered after plastic adherence as described previously (27). Collected cells had a viability >95%, as determined by trypan blue exclusion, and were CD11b$^+$ for ~90%, as determined by flow cytometry analysis (data not shown).

FACS staining

Cells were stained for 20 min at 4°C using conventional protocols. Cells were incubated with anti-FcγR Ab (clone 2.4G2) before adding (1 μg/10^6 cells): FITC/PE-conjugated anti-CD11b (clone M1/70), PE-conjugated anti-GR-1 (Ly6C/G, clone RB6-8C5), PE-conjugated anti-CD3 (clone 145-2C11), PE-conjugated anti-CD19 (clone 1D3), PE-conjugated anti-F4/80 (clone CI:A3-1), or PE-conjugated irrelevant control Abs (clone G155-178). All Abs were purchased from BD Biosciences, with the exception of anti-F4/80 (Serotec). Cells (10^4) were analyzed on a FACSVantage SE flow cytometer (BD Biosciences) using CellQuest program.

Quantification of NO_2 and arginase activity

Peritoneal cells (2×10^5/200 μl) were cultured in humidified atmosphere containing 5% CO_2 in complete medium in the absence or the presence of LPS (0.1 μg/ml, *Escherichia coli* O55:B5) for 48 h at 37°C. NO in culture supernatants was estimated by quantifying NO_2 using Greiss reagent as described previously (27). Arginase activity was measured in peritoneal cell lysates as described previously (32).

RNA extraction and quantitative RT-PCR analysis

One microgram of total RNA prepared using TRIzol reagent (Invitrogen Life Technologies) was reverse-transcribed using oligo(dT) and SuperScript II reverse transcriptase (Invitrogen Life Technologies) following the manufacturer's recommendations. Quantitative real-time PCR was performed in a Bio-Rad iCycler, with Bio-Rad iQ SYBR Green Supermix using as primers: ribosomal protein S12 sense (5′-CCTCGATGACATC CTTGGCCTGAG-3′) and antisense (5′-GGAAGGCATAGCTGCTG GAGGTGT-3′); arginase 1 sense (5′-ATGGAAGAGACCTTCAGCTAC-3′) and antisense (5′-GCTGTCTTCCCAAGAGTTGGG-3′); found in inflammatory zone 1 (FIZZ1) sense (5′-TCCCAGTGAATACTGATGA GA-3′) and antisense (5′-CCACTCTGGATCTCCCAAGA-3′); Ym sense (5′-GGGCATACCTTTATCCTGAG-3′) and antisense (5′-CCACTG AA GTCATCCATGTC-3′); MGL2 sense (5′-GATAACTGGCATGGACAT ATG-3′) and antisense (5′-TTTCTAATCACCATAACACATTC-3′); and 12/15-LOX sense (5′-CAGCTGGATTGGTTCTACTG-3′) and antisense (5′-CTCTGAACTTCTTCAGCACAG-3′). For all primers, each PCR cycle consisted of 1-min denaturation at 94°C, 45 s annealing at 55°C, and 1-min extension at 72°C. Gene expression was normalized using ribosomal protein S12 as housekeeping gene. Similar results were obtained using other housekeeping genes.

Proliferative assay

Single cell suspensions of mesenteric, axillary, and inguinal lymph nodes from noninfected mice (2×10^5) were cocultured with adherent peritoneal cells (at a ratio of 5%) and activated with Con A (2.5 μg/ml; Sigma-Aldrich) or anti-CD3 Ab (clone 145-2C11, 1 μg/ml; BD Biosciences) in 200 μl of complete medium. When required, the following inhibitors were used: N^G-monomethyl L-arginine (L-NMMA) (1000 μM; Sigma-Aldrich), *N*(omega)-hydroxy-nor-L-arginine (nor-NOHA) (250 μM; Bachem), superoxide dismutase (SOD) (200 U/ml; Sigma-Aldrich), catalase (300 U/ml; Sigma), nordihydroguaiaretic acid (NDGA) (10 μM; Sigma-Aldrich), GW9662 (10 μM; Sigma-Aldrich), and indomethacin (10 μg/ml). Stock solutions of NDGA, GW9662, and indomethacin were made in DMSO. Therefore, a corresponding volume of DMSO was added to cocultures performed in the absence of these inhibitors. After 24 h at 37°C in a humidified atmosphere containing 5% CO_2, cells were pulsed for 18 h with 1 μCi of [*methyl*-^{3}H]-thymidine ([^{3}H]Thy) (TRA310; Amersham Biosciences) and harvested onto glass fiber filters, and the incorporated radioactivity was measured in a liquid scintillator counter (1450 microbeta; Wallac). Results were expressed as the [^{3}H]Thy incorporation from triplicate cultures subtracting background values from nonstimulated cells. Proliferative responses (mean cpm ± SD of three mice tested individually) were compared in cocultures containing peritoneal cells from infected and noninfected mice.

Cytokine quantification

Cytokines were quantified using sandwich ELISAs for IFN-γ and IL-4 performed as recommended by the supplier (BD Biosciences). Maximal levels of cytokines observed after 3 days (IFN-γ) or 1 day (IL-4) of culture are reported.

Statistical analysis

All comparisons were tested for statistical significance ($p < 0.05$) via the unpaired *t* test, using GraphPad Prism 3.0 software.

Results

CD11b$^+$GR-1$^+$ myeloid cells are induced on T. crassiceps *infection*

Implantation of *T. crassiceps* in the peritoneal cavity of BALB/c mice caused a massive accumulation of cells in this compartment (Fig. 1). The cell number gradually increased, reaching a maximum 3–4 wk postinfection of $63.5 \pm 11 \times 10^6$ cells, as compared with $3.6 \pm 1.8 \times 10^6$ cells in noninfected animals. The cell number then decreased and stabilized at ~20×10^6 between the 6th and 12th wk of infection, time at which the experiment was stopped.

The modulation of the total cell number in the peritoneal cavity of *T. crassiceps*-infected animals was paralleled by a gradual increase in the percentage of CD11b$^+$ myeloid cells, from 38 ± 5% in noninfected mice to ~75% in late stage-infected animals (Fig.

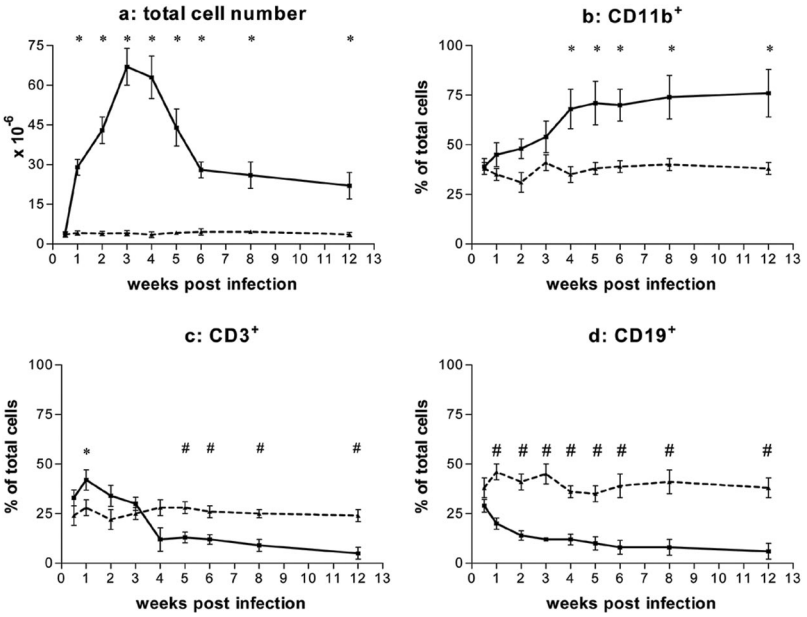

FIGURE 1. *T. crassiceps* infection causes CD11b$^+$ cell accumulation in the peritoneal cavity. At indicated time points postinfection, the total cell number (*a*) recovered from the peritoneal cavity of three infected mice was determined using a hemocytometer and compared with the cell number in two noninfected animals. In parallel, percentages of CD11b$^+$ (*b*), CD3$^+$ (*c*), and CD19$^+$ cells (*d*) in the total cell population were determined by flow cytometry. At each time point postinfection investigated, the mean ± SD of infected (■) and noninfected (▲) mice was compared. One representative of three independent experiments is shown. ∗, $p < 0.05$ higher comparing infected vs noninfected mice. #, $p < 0.05$ lower comparing infected vs noninfected mice.

1). Initially, the percentage of CD3$^+$ T cells increased, reaching a maximum of 42 ± 5% 1 wk after *T. crassiceps* implantation, as compared with the 24 ± 8% observed in noninfected mice, but became lower than in noninfected animals from the fourth week of infection. The percentage of CD19$^+$ B cells dropped very rapidly, from 38 ± 8% of the total cell population in noninfected mice to <5% in the late stage-infected mice.

Further characterization of the phenotype of peritoneal CD11b$^+$ cells revealed that the percentage of mature macrophages, coexpressing high levels of CD11b and F4/80 (33), tended to decrease upon *T. crassiceps* infection, in particular from 4–5 wk postinfection (Fig. 2). In contrast, the percentage of CD11b^{low}F4/80^{low} cells increased (see Fig. 4). In addition, CD11b^{low} cells that emerged gradually in the peritoneal cavity of infected mice coexpressed the myeloid epitope GR-1 (Fig. 2) at low or high levels (see Figs. 4 and 8). These CD11b^{low}GR-1^{low} and CD11b^{low}GR-1^{high} populations displayed low and high scatters, respectively (data not shown), likely reflecting the expansion of immature myeloid cells and granulocytes (33) in *T. crassiceps*-infected mice. May-Grunwald Giemsa staining of the GR-1^{low} and GR-1^{high} cells sorted from MACS-purified CD11b$^+$ peritoneal cells confirmed their nature as macrophage-like cells and granulocytes, respectively (Fig. 2). A CD11b^{low}GR-1$^-$ population decreasing gradually in the course of infection (see Figs. 4 and 8) possibly represented B cells (34).

Collectively, although the percentage of T and B cells decreased gradually, the proportion of CD11b$^+$ cells expanded up to two times in the peritoneal cavity of *Taenia*-infected animals as the disease progressed. Concomitantly, the percentage of CD11b^{high} F4/80^{high} mature macrophages tended to decrease, whereas the

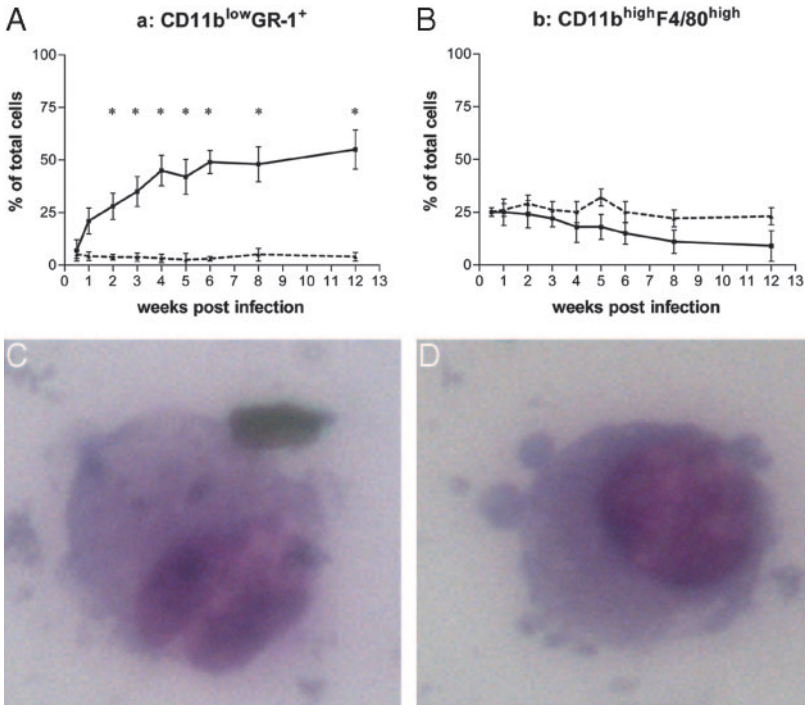

FIGURE 2. CD11b$^+$GR-1$^+$ cells expand in the peritoneal cavity of *T. crassiceps*-infected mice. At indicated time points postinfection, percentages of CD11b^{low}GR-1$^+$ (*A*) and CD11b^{high}F4/80^{high} cells (*B*) in the total cell population were determined by flow cytometry, as described in Fig. 1, using the gates depicted in Fig. 4 (■, infected mice; ▲, noninfected mice). ∗, $p < 0.05$ higher comparing infected vs noninfected mice. CD11b$^+$GR-1^{low} (*C*) and CD11b$^+$GR-1^{high} (*D*) cells were sorted from the peritoneal cavity of infected mice (2 wk postinfection) and stained with May-Grunwald Giemsa (×400).

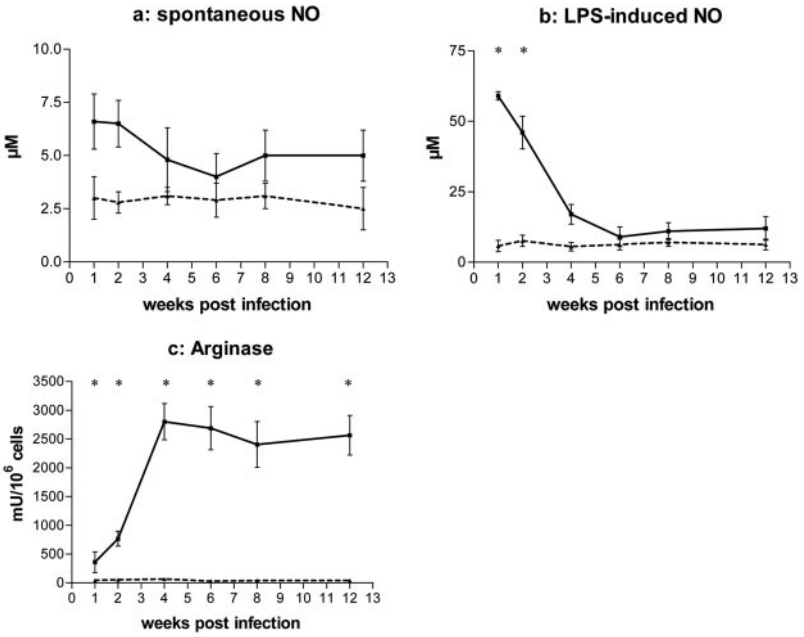

FIGURE 3. NO production and arginase activity in the peritoneal cavity of *T. crassiceps*-infected mice display reciprocal kinetics. At indicated time points postinfection, NO production was quantified in supernatants of peritoneal cells from three infected mice and two noninfected animals cultured in the absence (*a*) or the presence of LPS (*b*). In parallel, arginase activity was determined in peritoneal cell lysates (*c*). At each time point postinfection investigated, the mean ± SD of infected (■) and noninfected animals (▲) was compared. One representative of three independent experiments is shown. *, $p < 0.05$ higher comparing infected vs noninfected mice. #, $p < 0.05$ lower comparing infected vs noninfected mice.

percentage of CD11b^{low} cells likely consisting of immature myeloid cells (GR-1^{low}) and granulocytes (GR-1^{high}) increased up to ten times.

Alternative myeloid cells emerge gradually in the course of T. crassiceps *infection*

T. crassiceps-infected mice mount a mixed Th1/Th2 cytokine response in the early stage of infection that shifts to a more polarized Th2 cytokine response in the late phase of the disease due to a decrease in IFN-γ production (28, 35, 36). Considering that Th1 and Th2 cytokines antagonistically regulate the development of classical and alternative myeloid cells, the NO/arginase balance was investigated in *T. crassiceps*-infected mice. As reported previously (27), peritoneal cells from infected mice were sensitized to secrete higher amounts of NO than equivalent cells from noninfected mice only in the early stage of infection, in particular upon LPS stimulation (Fig. 3). In contrast, arginase activity was evidenced from the first week of infection and increased as the infection progressed to reach maximal values ~4 wk postinfection. NO secretion as well as arginase activity were confined to the adherent fraction of peritoneal cells (data not shown). Thus, we focused on this cell population in the work described below. Data from representative experiments performed in the early (<4 wk) and late (>6 wk) stage of infection are illustrated.

In the adherent peritoneal cell fraction, a gradual increase in CD11b^{low} cells coexpressing GR-1^{low} and/or F4/80^{low} was observed in the course of *T. crassiceps* infection as compared with noninfected mice (Fig. 4 and Table I). The percentage of CD11b^{low}GR-1^{high} cells also increased but only in the early stage of infection. The percentage of mature macrophages, i.e., CD11b^{high} cells expressing F4/80^{high} and being GR-1^{-}, did not differ significantly in the adherent cell fraction of infected and noninfected mice at the times investigated postinfection, and, if anything, it tended to be lower in infected animals. Finally, the percentage of adherent CD11b^{low} cells being GR-1^{-} or F4/80^{-} was lower in infected than in noninfected mice. Thus, mainly

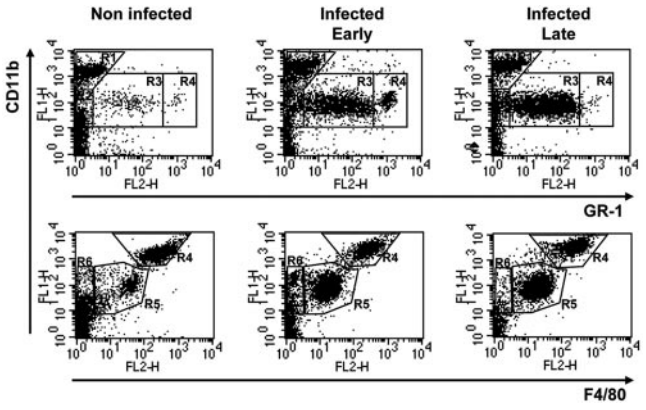

FIGURE 4. Adherent peritoneal cells from *T. crassiceps*-infected mice are enriched in CD11b^{low}GR-1^{+} cells. Adherent cell populations collected in the early (<4 wk) and late (>6 wk) stage of infection were stained with FITC-labeled anti-CD11b and PE-labeled anti-GR-1 or PE-labeled anti-F4/80 Abs. Simultaneously, cells from three noninfected mice were pooled and submitted to the same staining procedure. The percentage of gated positive cells was determined. Profiles are representative of one pool of cells from noninfected mice and of cells from one infected individual of seven investigated at each stage of infection in three independent experiments.

Table I. *Percentage of GR-1^{+} and F4/80^{+} cells within adherent CD11b^{+} peritoneal cells collected in wild-type mice in the early and late stage of* T. crassiceps *infection*[a]

		Stage of Infection	
	Noninfected	Early (<4 wk)	Late (>6 wk)
CD11b^{high}GR-1^{-} (R1)	30.3 ± 4.1	28.0 ± 4.5	24.6 ± 3.8
CD11b^{low}GR-1^{-} (R2)	15.3 ± 3.1	8.96 ± 2.1[b]	7.84 ± 2.1[b]
CD11b^{low}GR-1^{low} (R3)	6.3 ± 1.0	38.1 ± 5.0[c]	54.3 ± 6.0[c,d]
CD11b^{low}GR-1^{high} (R4)	0.78 ± 0.3	6.22 ± 2.1[c]	1.1 ± 1.1
CD11b^{high}F4/80^{high} (R5)	35.4 ± 4.1	20.4 ± 2.9[b]	27.5 ± 4.0
CD11b^{low}F4/80^{low} (R6)	13.6 ± 2.6	44.6 ± 4.1[c]	57.6 ± 4.3[c]
CD11b^{low}F4/80^{-} (R7)	14.7 ± 4.1	8.56 ± 2.6	4.6 ± 2.9[b]

[a] The percentage of cells within the gates illustrated in Fig. 4 was established. The mean ± SD of two pools of cells from both three noninfected mice and six infected mice is shown.

[b] $p < 0.05$ lower comparing infected vs noninfected mice.

[c] $p < 0.05$ higher comparing infected vs noninfected mice.

[d] $p < 0.05$ higher comparing early and late stage infected mice.

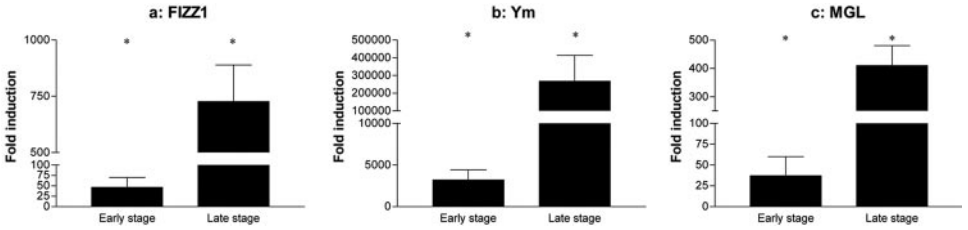

FIGURE 5. Modulation of FIZZ1, Ym, and MGL mRNA expression in adherent peritoneal cells from *T. crassiceps*-infected mice. Gene expression of FIZZ1 (*a*), Ym (*b*), and MGL (*c*) was determined via quantitative real-time PCR and normalized for the housekeeping gene ribosomal protein S12 in adherent peritoneal cells from infected mice collected in the early (< 4 wk) and late stage of infection (>6 wk). The fold induction of the gene expression (mean ± SD of three individual animals) in infected as compared with noninfected mice is shown for one representative of three independent experiments. *, $p < 0.05$ higher comparing infected vs noninfected mice.

$CD11b^{low}GR\text{-}1^{low}$ ($F4/80^{low}$) immature myeloid cells were enriched in the adherent fraction of peritoneal cells from *T. crassiceps*-infected mice as compared with noninfected animals.

Besides arginase, the expression of the genes coding for FIZZ1, Ym, and the macrophage galactose-type C-type lectin (MGL) family (37–39) is up-regulated in alternatively activated macrophages elicited during parasite infections. Expression levels of these genes were thus analyzed by real-time PCR to further address the activation status of adherent peritoneal cells in *T. crassiceps*-infected mice. As compared with noninfected mice, FIZZ1, Ym, and MGL mRNA expression gradually increased in the course of infection (Fig. 5). The up-regulation of the expression of these genes occurred as early as 4 days postinfection (data not shown).

In agreement with a previous report (27), adherent peritoneal cells from early stage-infected mice triggered the production of both IFN-γ and IL-4 when cocultured with lymph node cells from noninfected mice activated with Con A, whereas cells from late stage-infected animals induced only the release of IL-4 (Table II).

Collectively, these data suggest that $CD11b^{low}GR\text{-}1^{+}F4/80^{low}$ myeloid cells displaying characteristics of classical and alternative activation status developed in the early stage of *T. crassiceps* infection. Yet, a polarization toward alternative myeloid cells occurred gradually as the infection progressed. The change in the activation status further correlated with the capacity of myeloid cells from early or late stage-infected mice to elicit a mixed Th1/Th2 or a Th2 cytokine profile, respectively.

iNOS and arginase contribute differentially to the suppressive activity of classical and alternative myeloid cells

Previous studies have documented the existence of a profound T cell unresponsiveness in *T. crassiceps*-infected mice (35, 40–42). Because classical as well as alternative $CD11b^{+}GR\text{-}1^{+}$ myeloid cells have been implicated in immunosuppression, the ability of adherent cells from the peritoneal cavity of mice implanted with *T. crassiceps* to impair the proliferation of T cell from noninfected animals was investigated in the presence of the NO inhibitor L-NMMA and the arginase inhibitor nor-NOHA (Fig. 6).

In the early stage of infection, L-NMMA nearly completely reversed the suppressive activity of cells from *T. crassiceps*-infected mice on Con A-induced T cell proliferation (by 86 ± 14%). At this stage, nor-NOHA could not restore proliferation, despite the fact that cells from infected mice exerted significant arginase activity (Fig. 3).

In the late stage of infection, the suppressive activity exerted by adherent peritoneal cells from *Taenia*-infected mice on Con A-induced T cell proliferation was not affected by L-NMMA and was marginally reduced by nor-NOHA (by 28 ± 7%) (Fig. 6). Noteworthy at this stage, the combination of the two inhibitors almost fully restored the ability of T cells to proliferate (by 88 ± 12%).

Recent studies have shown that alternative MSC exert their suppressive activity through oxidative stress and that arginase activity

Table II. *IFN-γ and IL-4 production in supernatants of Con A-activated lymph node cells from noninfected mice cocultured with adherent peritoneal cells from* T. crassiceps*-infected animals*[a]

	Stage of Infection	IFN-γ (pg/ml)	IL-4 (pg/ml)
Noninfected		2567 ± 543	8 ± 4[b]
Infected	Early (<4wk)	8755 ± 1420[c]	230 ± 57[c]
Infected	Late (>6wk)	2345 ± 429	300 ± 70[c]

[a] For each experimental group, the mean ± SD of three mice of one representative of three independent experiments is shown.
[b] Below the detection limit of the cytokine quantification test (25 pg/ml).
[c] $p < 0.05$ higher comparing infected vs noninfected mice.

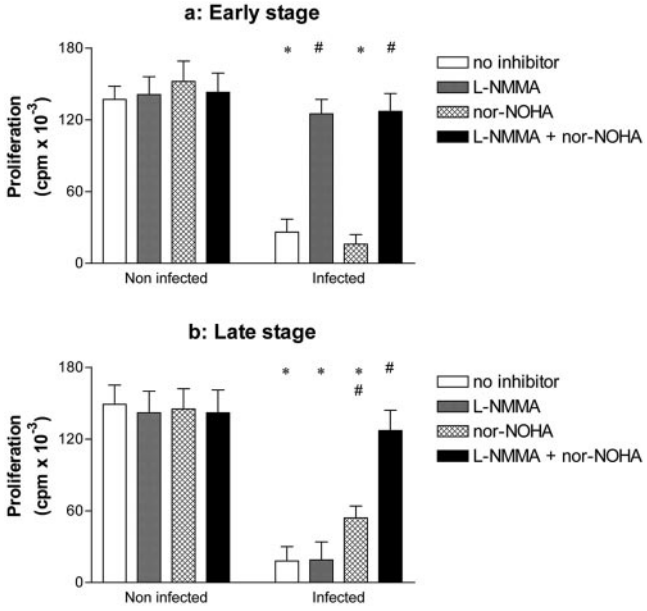

FIGURE 6. NO and arginase contribute to the antiproliferative capacity of adherent peritoneal cells from *T. crassiceps*-infected mice. Adherent peritoneal cells from three infected and three noninfected mice were cocultured with lymph node cells from noninfected mice activated with Con A in the absence or presence of NO (L-NMMA) and/or arginase (nor-NOHA) inhibitor. Proliferation was determined by [^{3}H]Thy incorporation. The mean ± SD of infected and noninfected mice, of one representative of three independent experiments performed in the early (<4 wk) (*a*) and late (>6 wk) (*b*) stage of infection, is shown. *, $p < 0.05$ lower comparing infected vs noninfected mice. #, $p < 0.05$ higher comparing infected mice in the presence vs the absence of inhibitor.

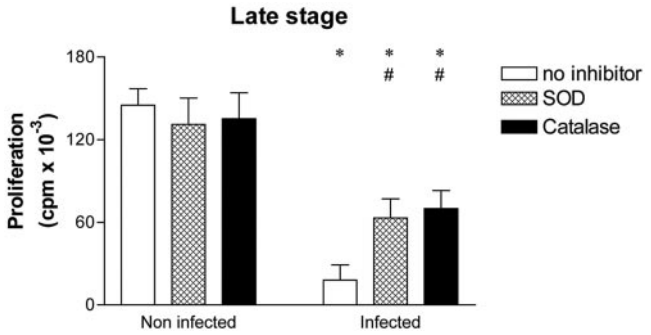

FIGURE 7. Superoxide and H_2O_2 contribute to the antiproliferative capacity of adherent peritoneal cells from late stage *T. crassiceps*-infected mice. Adherent peritoneal cells from three infected and three noninfected mice were cocultured with lymph node cells from noninfected mice activated with Con A in the absence or presence of superoxide (SOD) or H_2O_2 (catalase) inhibitor. Proliferation was determined by [^{3}H]Thy incorporation. The mean ± SD of infected and noninfected mice, of one representative of three independent experiments performed in the late stage of infection (> 6 wk), is shown. *, $p < 0.05$ lower comparing infected vs noninfected mice. #, $p < 0.05$ higher comparing infected mice in the presence vs the absence of inhibitor.

is the major contributor to the production of ROS (23, 32). Therefore, the potential role of ROS in the inhibition of T cell proliferation triggered by adherent alternative myeloid cells elicited in the late stage of *T. crassiceps* infection was evaluated (Fig. 7). SOD and catalase were found to restore partially but significantly the proliferation of T cell induced by Con A (by 41 ± 10% and 45 ± 10%, respectively). Hence, superoxide and H_2O_2 take part in the suppressive activity of alternative MSC elicited during *T. crassiceps* infection.

Together, these data suggest that in the early stage of *T. crassiceps* infection, classical MSC are responsible for the inhibition of T cell proliferation mainly via the secretion of NO. Yet, in the late stage of infection, alternative MSC block the proliferation in an arginase and iNOS-dependent pathway. In the whole course of the disease, the suppressive activity was reversed when the suppressive cell population was separated from responder T cells in transwell culture chambers. In addition, paraformaldehyde-fixed cells cocultured with responder cells fully retained their suppressive activity (data not shown). Therefore, as in *B. malayi*-infected mice (25), the suppressive activity in *T. crassiceps*-infected animals seems to be mediated by a direct cell contact. However, we cannot exclude that small molecules including NO and ROS still diffused through the membrane of fixed suppressive cells.

IL-4 and IL-13 trigger the expansion of alternative MSC

To address whether the induction of suppressive cells in the late stage of *T. crassiceps* infection depends on IL-4 and/or IL-13, experiments were performed in IL-4$^{-/-}$ and IL-R4$\alpha^{-/-}$ mice. Parasite burdens in peritoneal lavages were similar in infected wild-type, IL-4$^{-/-}$, and IL-R4$\alpha^{-/-}$ mice at the time when cells were isolated (data not shown). In agreement with previous reports (25, 35), no suppressive activity of adherent peritoneal cells on T cell proliferation triggered by Con A was recorded in the absence of IL-4 (IL-4$^{-/-}$ mice) or of IL-4 and IL-13 (IL-4R$\alpha^{-/-}$ mice) (data not shown). Correspondingly, real-time PCR analyses revealed that arginase mRNA expression in adherent peritoneal cells was not induced upon infection in IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice, in contrast to wild-type animals (Table III). In addition, the induction of FIZZ1 and MGL expression was abolished in the absence of IL-4 and/or IL-13 signaling in infected mice. Finally, the induction of Ym expression observed in infected wild-type animals was lowered drastically in IL-4$^{-/-}$ and even more in IL-4R$\alpha^{-/-}$-infected mice.

The contribution of IL-4 and IL-13 to the accumulation of CD11b$^+$GR-1$^+$ cells in *T. crassiceps*-infected mice was investigated in the late stage of infection (Fig. 8 and Table IV). In noninfected wild-type, IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice, the percentages of CD11b$^+$ populations, including GR-1$^-$, GR-1^{low}, and GR-1^{high} cells, were similar. In infected animals, the percentages of CD11b$^+$ cells, as compared with wild-type mice, slightly decreased in IL-4$^{-/-}$ mice and were significantly lower in IL-4R$\alpha^{-/-}$ mice. Notably, the percentages of the GR-1$^-$ cells expressing high or low levels of CD11b, and of CD11b^{low}GR-1^{low} cells, were significantly lower in infected IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice than in infected wild-type mice. In contrast, the percentage of CD11b^{low}GR-1^{high} cells increased both in infected IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice. Finally, a population of CD11b$^-$GR-1^{low} cells, which was marginally present in the three strains of noninfected mice and in infected wild-type mice, expanded in IL-4$^{-/-}$ and IL-4R$\alpha^{-/-}$ mice. The decrease in CD11b^{high}GR-1$^-$, CD11b^{low}GR-1$^-$, and CD11b^{low}GR-1^{low} cells concomitant with the increase in CD11b^{low}GR-1^{high} and CD11b$^-$GR-1^{low} cells already occurred in the early stage of infection (data not shown).

In accordance with a previous report (35), the populations expressing GR-1 that expanded in the absence of IL-4 and or IL-13 signaling in *Taenia*-infected mice should not be neutrophils. Indeed, the percentage of peritoneal myeloperoxidase-positive cells (cytochemical localization) and the gene expression levels of myeloperoxidase in adherent peritoneal cells (real-time PCR) were similar in knockout (KO) and wild-type mice both in the early and the late stage of infection (data not shown). Although not firmly

Table III. *Modulation of arginase, FIZZ1, Ym, MGL, and 12/15-LOX mRNA expression in adherent peritoneal cells of wild-type, IL-4$^{-/-}$, and IL-4R$\alpha^{-/-}$ mice in the late stage of* T. crassiceps *infection*[a]

	Noninfected			Infected		
	Wild Type	IL-4$^{-/-}$	IL-4R$\alpha^{-/-}$	Wild Type	IL-4$^{-/-}$	IL-4R$\alpha^{-/-}$
Arginase	1.00 ± 0.51	1.11 ± 0.60	1.21 ± 0.32	67.31 ± 1.68[b]	3.0 ± 1.1[c]	1.7 ± 1.3[c]
FIZZI	1.00 ± 0.20	1.09 ± 0.38	1.30 ± 0.47	597 ± 103[b]	1.51 ± 1.25[c]	0.01 ± 0.008[c,d,e]
Ym	1.00 ± 0.06	1.20 ± 0.28	1.25 ± 0.40	219487 ± 143386[b]	353 ± 240[b,c]	26.3 ± 11.96[b,c,e]
MGL	1.00 ± 0.19	1.19 ± 0.51	1.20 ± 0.38	406 ± 98[b]	0.99 ± 0.67[c]	0.04 ± 0.01[c,d,e]
12/15-LOX	1.00 ± 0.88	1.47 ± 0.74	1.74 ± 0.16	0.77 ± 0.67	0.01 ± 0.004[c,d]	0.01 ± 0.004[c,d]

[a] The gene expression was determined via quantitative real-time PCR and normalized for the housekeeping gene ribosomal protein S12. The fold induction (mean ± SD of three individual animals) of the gene expression in adherent peritoneal cells as compared to noninfected wild-type mice is shown. Data are representative of one of two independent experiments performed in the late stage of infection (>6 wk).

[b] $p < 0.05$ higher comparing infected vs noninfected mice.

[c] $p < 0.05$ lower comparing infected KO mice vs infected wild-type mice.

[d] $p < 0.05$ lower comparing infected vs noninfected mice.

[e] $p < 0.05$ lower comparing infected IL-4R$\alpha^{-/-}$ vs infected IL-4$^{-/-}$ mice.

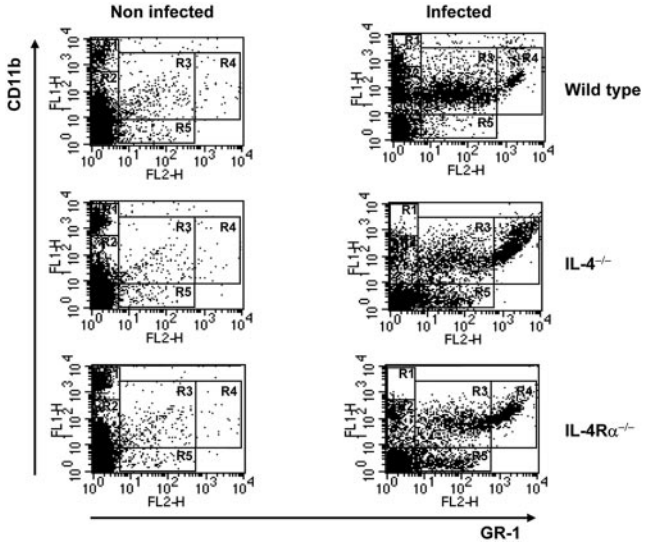

FIGURE 8. IL-4 and/or IL-13 contribute to the expansion of $CD11b^+GR\text{-}1^+$ cells in the peritoneal cavity of late stage *T. crassiceps*-infected mice. In the late stage of infection (>6 wk), cells from the peritoneal cavity were collected in individual wild-type, IL-$4^{-/-}$, and IL-$4R\alpha^{-/-}$ mice and stained with FITC-labeled anti-CD11b and PE-labeled anti-GR-1 Abs. Simultaneously, cells from three noninfected mice were pooled and submitted to the same staining procedure. The percentage of gated positive cells within the total cell population was determined. Profiles are representative of one pool of cells from noninfected mice and of cells from one infected individual of six investigated in two independent experiments.

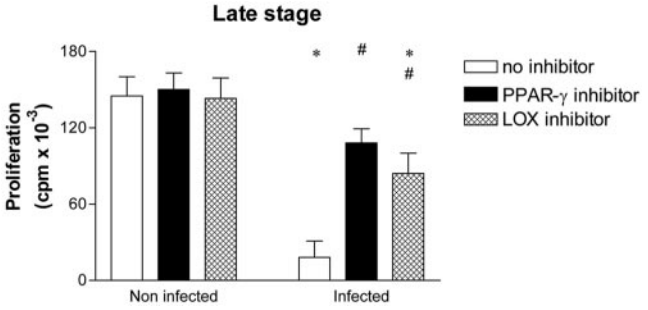

FIGURE 9. The 12/15-LOX and PPAR-γ activation contribute to the antiproliferative capacity of adherent peritoneal cells from late stage *T. crassiceps*-infected mice. Adherent peritoneal cells from three infected and three noninfected mice were cocultured with lymph node cells from noninfected mice activated with Con A in the absence or presence of NDGA (LOX inhibitor) or GW9662 (PPAR-γ inhibitor). Proliferation was determined by [^{3}H]Thy incorporation. The mean $\pm$ SD of infected and noninfected mice of one representative of three independent experiments performed in the late stage of infection (>6 wk) is shown. *, $p < 0.05$ lower comparing infected vs noninfected mice. #, $p < 0.05$ higher comparing infected mice in the presence vs the absence of inhibitor.

established, these expanding $GR\text{-}1^+$ populations may represent eosinophils (33) and/or B cells (34).

12/15-LOX activity contributes to the suppressive activity of alternative myeloid cells

IL-4 has been reported to interfere with T cell proliferation by inducing the macrophage 12/15-LOX activity, hereby generating potential ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ), a member of the ligand-dependent nuclear receptor family (43). To assess this possible pathway of suppression, *12/15-LOX* gene expression was evaluated by real-time PCR in adherent peritoneal cells collected in the late stage of *T. crassiceps* infection. As shown in Table III, *12/15-LOX* gene expression was similar in infected and noninfected wild-type animals. However, in infected IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice, the expression of 12/15-LOX mRNA was abolished completely.

Next, the suppressive activity of adherent peritoneal cells from late stage infection was investigated in the presence of NDGA, a general LOX inhibitor, or of GW9662, as a PPAR-γ antagonist. As shown in Fig. 9, the Con A-induced T cell proliferation was restored by the LOX or the PPAR-γ inhibitor (by 55 $\pm$ 9% or 62 $\pm$ 11%, respectively). Although ligands for PPAR-γ can derive from the cyclooxygenase (COX) cascade (44), the nonselective COX inhibitor indomethacin did not influence the suppressive activity of adherent peritoneal cells from *T. crassiceps*-infected mice (data not shown).

Together, these data indicate that IL-4/IL-13 contribute to the suppressive activity elicited during *T. crassiceps* infection through 12/15-LOX activation, resulting in the production of PPAR-γ ligands. This mechanism of suppression, as those induced by arginase and ROS, are only active in the late stage of infection (data not shown), i.e., when only alternative MSC are elicited.

It is worthwhile mentioning that, as with Con A, the inhibition of anti-CD3 Ab-induced T cell proliferation depended on iNOS for MSC elicited in the early stage of infection. In contrast, in the late stage of infection, arginase plus iNOS, superoxide, H_2O_2, LOX,

Table IV. *Percentage of $CD11b^+$ and $GR\text{-}1^+$ cells in the peritoneal cavity of wild-type, IL-$4^{-/-}$ and IL-$4R\alpha^{-/-}$ mice in the late stage of* T. crassiceps *infection*[a]

	Noninfected			Infected		
	Wild Type	IL-$4^{-/-}$	IL-$4R\alpha^{-/-}$	Wild Type	IL-$4^{-/-}$	IL-$4R\alpha^{-/-}$
$CD11b^+$ (R1–R4)	44.2 $\pm$ 9	36.5 $\pm$ 8	45.3 $\pm$ 7	66.4 $\pm$ 6[b]	51.1 $\pm$ 7[b]	34.3 $\pm$ 7[c,d]
$CD11b^{high}$ $GR\text{-}1^-$ (R1)	5.40 $\pm$ 2.6	9.1 $\pm$ 3.9	7.39 $\pm$ 2.8	3.51 $\pm$ 1.9	0.34 $\pm$ 0.36[e]	0.03 $\pm$ 0.03[e]
$CD11b^{low}$ $GR\text{-}1^-$ (R2)	25.9 $\pm$ 3.6	19.3 $\pm$ 4.9	28.4 $\pm$ 5.8	22.0 $\pm$ 1.9	8.21 $\pm$ 1.66[e]	7.64 $\pm$ 2.03[e]
$CD11b^{low}$ $GR\text{-}1^{low}$ (R3)	3.89 $\pm$ 1.2	2.67 $\pm$ 1.4	3.0 $\pm$ 1.4	30.2 $\pm$ 4[b]	17.2 $\pm$ 3.1[b,c]	14.8 $\pm$ 4.1[b,c]
$CD11b^{low}$ $GR\text{-}1^{high}$ (R4)	0.50 $\pm$ 0.30	0.36 $\pm$ 0.28	0.24 $\pm$ 0.19	6.10 $\pm$ 1.1[b]	23.4 $\pm$ 1.9[b,c]	11.01 $\pm$ 2.4[b,f]
$CD11b^-$ $GR\text{-}1^+$ (R5)	1.77 $\pm$ 1.8	1.57 $\pm$ 1.5	1.24 $\pm$ 1.8	1.69 $\pm$ 1.6	9.37 $\pm$ 2.4[b,c]	9.27 $\pm$ 1.9[b,f]

[a] The percentage of cells within the gates illustrated in Fig. 7 was established. The mean $\pm$ SD of two pools of cells from three noninfected mice and of six infected mice is shown.

[b] $p < 0.05$ higher comparing infected vs noninfected mice within each mouse strain.

[c] $p < 0.05$ lower comparing infected KO mice vs infected wild-type mice.

[d] $p < 0.05$ lower comparing infected IL-$4R\alpha^{-/-}$ mice vs infected IL-$4^{-/-}$ mice.

[e] $p < 0.05$ lower comparing infected vs noninfected mice within each mouse strain.

[f] $p < 0.05$ higher comparing infected KO mice vs infected wild-type mice.

and PPAR-γ contributed to the impairment of TCR-triggered T cell proliferation caused by alternative MSC (data not shown).

Discussion

The helminth *Taenia solium* causes neurocysticercosis in humans and pigs. This disease is a major public health problem in South America and Asia, and the number of reported cases in developed countries is increasing currently (45). In experimental models of cysticercosis, *T. crassiceps*-infected mice gradually shift from an early Th1/Th2 immune response to a Th2-biased immune response in the late stage of the disease. As one of the exceptions to the canonical believe that a Th2 immune response protects against helminth infections, a Th1 immune response is essential for host defense against *T. crassiceps*. In this parasitic disease, the Th2-biased immune response is associated with an increase in parasite load (27, 35, 46).

Recent studies suggest that a subset of macrophages called alternatively activated macrophages play a role in the bias toward the Th2 immune response induced by *T. crassiceps* or other helminths. Concomitantly, they weaken the host immune status by suppressing helminth-related and -unrelated immune responses. Such immunosuppression was proposed to impair the induction of the Th1 immune response, to increase the susceptibility of both mice and human to other diseases, or to limit the efficacy of vaccination in infected populations (25, 27, 47–51). In this study, we have further investigated the phenotype and mechanisms by which cells from the peritoneal cavity of mice implanted with *T. crassiceps* down-regulate T cell proliferation.

In accordance with previous microscopic analyses (26, 27, 35), we observed that as *T. crassiceps* infection progresses, a gradual decrease in the percentage of T and B cells and an increase in the percentage of $CD11b^{low}GR\text{-}1^{high}$ cells representing granulocytes occurs. We also document that the percentage of $F4/80^{high}CD11b^{high}$ mature macrophages tends to decrease. Concomitantly, a population of $CD11b^{low}$ $GR\text{-}1^{low}$ immature myeloid cells, which is marginally present in the peritoneal cavity of noninfected mice, expands gradually in the course of *Taenia* infection.

Adherent peritoneal cells from *T. crassiceps*-infected mice inhibit the proliferative response of T cells from noninfected mice triggered by Con A in a cell contact-dependent manner. The suppressive activity in this population, exhibiting in the course of infection an increase in $CD11b^{low}F4/80^{low}$ cells expressing different levels of GR-1 (high or low), could thus be due to granulocytes, immature myeloid cells, or both. We believe that $CD11b^{low}GR\text{-}1^{low}$ cells are the most likely suppressive cells. Indeed, their surface phenotype corresponds to the one of MSC, the suppressive activity of which was established clearly in various pathologies (20). Moreover, we document that the disappearance of suppressive activity in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ mice infected with *T. crassiceps* correlates with the loss of $CD11b^{low}GR\text{-}1^{low}$ cells and the expansion of $CD11b^{low}GR\text{-}1^{high}$ granulocytes.

The activation of peritoneal MSC in *T. crassiceps*-infected mice changes from a classical/alternative status in the early stage of infection to a predominant alternative status in the late stage of infection. The apoptosis of T and B cells observed in the early stage of *T. crassiceps* infection (data not shown and Ref. 52) as well as parasite-derived glycoconjugate and/or protein moieties may contribute to the emergence of alternative MSC, as described in other infection models (10, 53–58). Reflecting the change in the activation status, MSC from the early or late stage of infection trigger T cells to secrete Th1/Th2 or Th2 cytokines, respectively. In addition, mechanisms underlying their antiproliferative function modify in the course of infection. Early stage MSC, despite exhibiting iNOS and arginase activity, block T cell proliferation only through their secretion of NO. This is evocative of classical MSC induced in tumor- or *Trypanosoma cruzi*-induced suppression (13, 15, 19, 59). In the late stage of infection, alternative MSC from *T. crassiceps*-infected animals impair T cell proliferation via their arginase activity. Yet, the combination of arginase and NO inhibitors was required to fully restore the T cell proliferative response. Moreover, alternative MSC impair T cell proliferative response by producing superoxide and H_2O_2. These mechanisms are reminiscent of alternative MSC elicited in cancer models (22, 23, 32). The current working mechanism is that arginase activity, by depleting L-arginine in alternative MSC, forces the iNOS reductase activity. This ends in the production of ROS, including superoxide and H_2O_2 that impair T cell proliferation. It should be remarked that the iNOS inhibitor L-NMMA could contribute to the depletion of arginine in alternative MSC by competing with CAT-2B, the transporter of L-arginine within cells (60).

IL-4 and IL-13 support the expansion of early myeloid progenitors and are required for the recruitment of immune cells in lymphoid tissues (61–63). Fittingly, the present and other reports demonstrate that IL-4 and/or IL-13 signaling is required for the generation of suppressive cells in the peritoneal cavity of helminth-infected mice (25, 26, 29, 35, 38, 64). Our data show that both cytokines could contribute to the antiproliferative capacity of *T. crassiceps*-induced alternative MSC by triggering arginase gene expression. Moreover, IL-4/IL-13 may play a role in the suppressive activity of alternative MSC by allowing the expression of the *12/15-LOX* gene. The 12/15-LOX, previously associated with alternative macrophage activation (65), generates bioactive lipid mediators like 13-hydroxyoctadecadienoic acid and 15-hydroxyeicosatetraenoic acid, which serve as ligands for PPAR-γ. This nuclear hormone receptor, initially identified as a key mediator of lipid metabolism, exerts immunoregulatory functions at the level of both macrophages and T cells (66). When activated in T lymphocytes, PPAR-γ inhibits their proliferation by promoting the inhibition of IL-2 production (43, 67, 68). Accordingly, in *T. crassiceps*-infected animals, the inhibition of proliferation to Con A was shown to result from impaired IL-2 production (40, 42). In this article, we show that the suppressive capacity of alternative MSC is impaired partially by a LOX and PPAR-γ inhibitor. These observations support the idea that in *T. crassiceps* infection, IL-4/IL-13-activated alternative MSC exert 12/15-LOX activity, hereby secreting molecules that inhibit proliferation via PPAR-γ activation in T cells. In contrast, a COX inhibitor does not affect the suppressive potential of cells from *T. crassiceps*-infected mice. Thus, ligands of PPAR-γ like 15-deoxy-$\Delta^{12,14}$ prostaglandin. J2 that can be produced via the COX pathway (44) do not play a role in the antiproliferative activity of alternative MSC. Besides a role in the generation of PPAR-γ ligands, 12/15-LOX could also contribute to the production of ROS (69).

In summary, our study reveals the expansion of $CD11b^{low}GR\text{-}1^{low}$ MSC in the course of *T. crassiceps* infection in mice. Early stage MSC, displaying the characteristics of classical and alternative activation, impair T cell proliferation via NO secretion, whereas the suppressive activity of alternative MSC elicited in the late stage of infection depends on the production of ROS via arginase activity and the production of PPAR-γ ligands via 12/15-LOX activity. Finally, IL-4/IL-13 signaling contributes to the expansion and suppressive activity of MSC. Therefore, this work proposes new intervention strategies to reverse immunosuppression, hereby restoring the efficacy of vaccination and resistance to opportunistic diseases in helminth-infected hosts.

Disclosures

The authors have no financial conflict of interest.

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