

A Schistosome-Expressed Immunomodulatory Glycoconjugate Expands Peritoneal Gr1⁺ Macrophages That Suppress Naive CD4⁺ T Cell Proliferation Via an IFN- γ and Nitric Oxide-Dependent Mechanism

Olga Atochina,^{1*} Toby Daly-Engel,* Danuta Piskorska,[†] Edward McGuire,[†] and Donald A. Harn*

Lacto-*N*-fucopentaose III (LNFPIII) is found in human milk and on the Th2 driving helminth parasite *Schistosoma mansoni*. This pentasaccharide drives Th2-type responses in vivo and in vitro when conjugated to a carrier. In an attempt to further understand early events in Th1 to Th2 switching, we examined phenotypic and functional changes in peritoneal cell populations in BALB/c and SCID mice following LNFPIII-dextran injection. We found that i.p. injection with LNFPIII-dextran resulted in rapid (<20 h) expansion of the Gr1⁺ subpopulation of F4/80⁺/CD11b⁺ peritoneal cells, comprising up to 75% of F4/80⁺/CD11b⁺ peritoneal cells compared with 18% in uninjected or dextran-injected mice. Functionally, these cells suppressed anti-CD3- and anti-CD28-induced proliferation of naive CD4⁺ T cells. LNFPIII-dextran also expanded functional Gr1⁺ suppressor macrophages in SCID mice, demonstrating that expansion and function of suppressor cells did not require T cells. Suppression in both BALB/c and SCID mice was NO and IFN- γ dependent, as addition of inhibitors of inducible NO synthase (*N*^G-monomethyl-L-arginine), as well as anti-IFN- γ Abs, restored the ability of CD4⁺ T cells to proliferate in vitro. Depletion of the F4/80⁺ subset of Gr1⁺ cells eliminated the suppressive activity of peritoneal exudate cells showing that these cells were macrophages. Thus, LNFPIII-dextran rapidly expands the Gr1⁺ suppressor macrophage population in the peritoneal cavities of otherwise naive mice. These Gr1⁺ cells suppress proliferation of naive CD4⁺ T cells in an NO-dependent mechanism, and may play a regulatory role in the switching of Th1- to Th2-type responses. *The Journal of Immunology*, 2001, 167: 4293–4302.

The innate immune system allows cells to respond to infectious agents through the binding of membrane pattern recognition receptors to various classes of pathogen-associated molecular patterns (1). This initial innate response recruits and activates effector cells and helps direct the ensuing adaptive response to the pathogen (2). Innate responses to microbial pathogens are generally pro-inflammatory, and may include the release of IL-1, IL-6, IL-8, IL-12, IFN- γ , or TNF- α as well as pro-inflammatory chemokines (3). In contrast to Th1-type pro-inflammatory responses, chronic helminth infection leads to polarized Th2-type responses, and little is known about innate immune responses during acute helminth infection. However, it has been shown that soon after exposure to schistosome egg Ags, mice produce Th1 pro-inflammatory responses, which then switch to polarized Th2-type responses (4). The mechanisms that enable the immune response to switch from Th1 to Th2 type are not known, but likely involve one or more types of innate interactions. In this regard, recent studies have described an innate response that leads to expansion of suppressor macrophage populations (5–8). These immunoregulatory cells, termed natural suppressor (NS)² cells, originate from gran-

ulocyte-monocyte progenitors, express Gr1, CD11b, and/or F4/80 surface markers, and are capable of inhibiting proliferative responses of naive or activated T and B cells. That helminth infection may involve NS cells was suggested in a study where peritoneal implantation of larval filarial parasites in mice expanded the resident NS population (9–11).

All suppressor macrophages resemble one another phenotypically and share a similar suppressor function. Despite these similarities, mechanistically there are two different subpopulations of suppressor macrophages: 1) classically activated (CA) macrophages, which are IFN- γ dependent (12–15); and 2) alternatively activated (AA) macrophages, which are IL-4 dependent (3, 16–19). IFN- γ -dependent Gr1⁺ suppressors are found in the bone marrow and peripheral lymphoid organs in cancer patients or tumor-bearing mice, and during viral infection (12–14). AA macrophages are found in the peritoneal cavity (PC) of mice in response to the filarial nematode *Brugia malayi* as well as in other cases (3, 9–11, 20, 21).

In schistosome infection of mice or humans, parasite carbohydrates have been shown to play a role in driving Th2-type and/or anti-inflammatory responses (22). Two immunoregulatory oligosaccharides expressed on schistosomes have been described (23–25). Therefore, we examined early immune responses to the schistosome-expressed immunoregulatory oligosaccharide lacto-*N*-fucopentaose III (LNFPIII) in an attempt to elucidate how this oligosaccharide may contribute to T helper biasing. We examined phenotypically and functionally the peritoneal cell response in

*Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115; and [†]Neose Technologies, Horsham, PA 19044

Received for publication April 3, 2001. Accepted for publication August 16, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Address correspondence and reprint requests to Dr. Olga Atochina, Department of Immunology and Infectious Diseases, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115. E-mail address: oatochin@hsph.harvard.edu

² Abbreviations used in this paper: NS, natural suppressor; LNFPIII, lacto-*N*-fucopentaose III; PEC, peritoneal exudate cell; PC, peritoneal cavity; CA, classically

activated; AA, alternatively activated; L-nMMA, *N*^G-monomethyl-L-arginine; LNF-P-II-dex, LNFPIII conjugated to dextran; LNT-dex, lacto-*N*-neotetraose-dextran; MnTBAP, manganese [III] tetrakis [4-benzoic acid] porphyrin.

mice injected with a glycoconjugate composed of LNFPIII conjugated to dextran (LNFPIII-dex) and found that a single injection of LNFPIII-dex rapidly expanded the peritoneal CD11b⁺/Gr1⁺/F4/80⁺ population in a T cell-independent manner. These cells suppressed the proliferation of naive CD4⁺ T cells, and the process of inhibition was found to be NO- and IFN- γ -dependent. Depletion of Gr1⁺/F4/80⁺ cells from the peritoneal exudate cells (PECs) of LNFPIII-dex-injected mice eliminated their ability to suppress proliferation of naive T cells. In addition, injection of the structurally related glycoconjugate lacto-*N*-neotetraose-dextran (LNNt-dex) has also been shown to expand suppressor cells in the PCs of otherwise naive mice.³ The ability of these helminth-expressed oligosaccharides to rapidly expand this particular suppressor population may be one mechanism that schistosomes use to modulate the host response and induce immune energy.

Materials and Methods

Mice

Female BALB/c and SCID:SCID mice between 6 and 8 wk of age were used in these studies and were purchased from Taconic Farms (Germantown, NY) and The Jackson Laboratory (Bar Harbor, ME).

Media and reagents

LNFPIII-dex and dextran were obtained from Neose Technologies (Horsham, PA). The glycoconjugate consisted of 12 LNFPIII molecules conjugated to a 10-kDa molecule of dextran. We tested for the presence of endotoxin in LNFPIII-dextran using a RAW murine macrophage bioassay for the production of NO and IL-10. RAW cells were stimulated with LPS (5 μ g/ml) or LNFPIII-dextran (25 and 50 μ g/ml) for 24, 48, or 72 h after which supernatants were collected and NO and IL-10 levels were determined by Griess reagent or ELISA. Both IL-10 and NO were produced in response to LPS, whereas neither was detected in supernatants from LNFPIII-dextran-stimulated cells. RPMI 1640 medium was supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 100 U/mg penicillin, 100 μ g/ml streptomycin, 0.05 mM 2-ME, and 2 mM glutamine (Sigma, St. Louis, MO). L-nMMA (*N*^G-monomethyl-L-arginine) and MnTBAP (manganese [III] tetrakis [4-benzoic acid] porphyrin) were obtained from Calbiochem (La Jolla, CA).

Monoclonal Abs

Purified anti-mouse CD3e (clone 145-2C11), anti-CD28, anti-mouse IFN- γ , CD11b-FITC, Gr-1-PE (RB6-8C5), CD4-PE, purified rat IgG1, and IgG2a isotype control IgG were purchased from BD PharMingen (San Diego, CA). Rat anti-mouse F4/80-Cy5 mAb was purchased from Serotec (Raleigh, NC). Anti-FITC and anti-PE microbeads were obtained from Miltenyi Biotec (Auburn, CA).

Cell preparation

Mice were injected i.p. with 50 μ g of LNFPIII-dex or dextran in HBSS (Life Technologies, Rockville, MD). Approximately 20 h postinjection, mice were euthanized by CO₂ inhalation, and PECs were obtained by lavage under sterile conditions by injection of 5 ml of HBSS into the PC. In some experiments, Gr1⁺ and F4/80⁺ cells were separated from PECs of LNFPIII-dex- or dextran-injected mice. PECs were incubated for 30 min at 4°C with anti-F4/80-FITC and anti-Gr1-PE Abs. Cells were washed to remove unbound Abs then incubated with anti-FITC and/or anti-PE microbeads for 20 min at 4°C. Positive and negative cell populations were then separated on a MACS column according to the manufacturer's instructions. Purity of the various populations was determined via FACScan.

T cell activation and proliferation assay

Spleen cell preparations were prepared from naive mice. Following lysis of RBC with Boyle's solution, splenocytes were washed and resuspended at 5–10 \times 10⁶/ml in PBS. CFSE (Molecular Probes, Eugene, OR) was added to a final concentration of 5 μ M and incubated at room temperature for 8 min. Next, FBS was added to a final concentration of 20%. Cells were

washed three times in cold RPMI 1640/10% FBS and plated. CFSE-labeled splenocytes (at a concentration of 1 \times 10⁶/ml and a volume of 0.5 ml) were plated in 48-well plates coated with 1 μ g/ml anti-CD3 and 5 μ g/ml anti-CD28 mAbs. Splenocytes were cultured for 3 h on Ab-coated plates, then 0.5 ml of PECs was added such that the ratios of PECs to splenocytes/T cells were 1:2, 1:4, 1:8, and 1:16. The cocultures were incubated for 72 h, then harvested and analyzed by flow cytometry. In some experiments, L-nMMA, MnTBAP, or anti-IFN- γ mAbs (final concentrations 0.5 mM, 10 μ M, and 10 μ g/ml, respectively) were added to coculture of splenocytes and PECs. In the same experiments, cocultured supernatants were harvested and levels of NO measured by mixing equal volumes of culture supernatants (50 μ l) and Griess reagent. After a 5-min incubation at room temperature, the absorbance was read at 550 nm using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were determined by comparing absorbance values of the test samples to a standard curve generated by serial dilution of 62.5 μ M sodium nitrite.

Flow cytometry

FITC- or PE-labeled positive and negative populations of Gr1 and F4/80 PECs, isolated as described in *Cell preparation*, or CFSE-labeled cocultured cells (2–5 \times 10⁵) were transferred to 12 \times 75-mm polystyrene tubes and washed with FACS buffer (PBS containing 0.1% BSA and 0.1% sodium azide). Cocultured cells or freshly isolated PECs were stained with various combinations of mAbs for 30 min on ice in the dark and washed twice in FACS buffer. Acquisition of cells was performed using a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA). A minimum of 20,000 events was required for analysis. Cell populations were analyzed using CellQuest software (BD Biosciences).

Statistical analysis

Statistical significance of difference in values among groups was determined by Student's *t* test. All data are expressed as the mean \pm SE.

Results

Injection of LNFPIII-dex expands Gr1⁺ macrophages in the PCs of mice

BALB/c mice were used to study the expansion and function of PEC subpopulations within 20 h postinjection of 50 μ g of LNFPIII-dex or dextran alone. As shown in Fig. 1A, PECs from LNFPIII-dex-injected mice contained higher numbers of Gr1⁺/CD11b⁺ cells than control (uninjected and dextran-injected) mice. The presence of Gr1⁺/F4/80⁺ PECs in LNFPIII-dex-injected mice is shown in Fig. 1B. Analysis of double-positive cells from sugar-injected mice showed two subpopulations characterized as Gr1⁺ high or low (Fig. 1, arrows). Both Gr1⁺ subpopulations express CD11b and F4/80 surface markers. To study the expression of Gr1 by macrophages, we gated double-positive CD11b/F4/80 cells (Fig. 1C, *left panel*). The results, showing Gr1-positive cells, are given in the histogram in Fig. 1C (*right panel*).

The percentages of Gr1⁺/CD11b⁺ cells and Gr1⁺ cells of CD11b/F4/80-positive PECs are summarized in Table I. Injection of LNFPIII-dex resulted in a significant (*p* < 0.001) increase in the percentage of CD11b⁺/F4/80⁺ macrophages expressing the Gr1⁺ marker (75.55 \pm 2.1%) compared with control (uninjected and dex-injected mice, 18.48 \pm 1.83 and 18.39 \pm 1.88%, respectively). It is clear that not only the percentage but also the absolute number of Gr1⁺ macrophages was dramatically increased in mice injected with LNFPIII-dex.

PECs from mice injected with LNFPIII-dex conjugate suppress the proliferation of CD4⁺ T cells stimulated with anti-CD3 and anti-CD28

In several reports, Gr1⁺ CD11b- and/or F4/80-positive populations suppressed naive T cell proliferation to mitogen or costimulatory signaling. Here we performed experiments to determine whether Gr1⁺, F4/80⁺ PECs from LNFPIII-dex-injected mice were able to suppress proliferation of naive splenocytes. Naive

³ L. Terrazas, K. Walsh, D. Piskorska, E. McGuire, and D. Harn. Lacto-*N*-neotetraose expands Gr-1⁺ suppressor cells that secrete anti-inflammatory cytokines and inhibit proliferation of nave CD4⁺ cells: a potential mechanism for immune polarization in helminth infections. *Submitted for publication*.

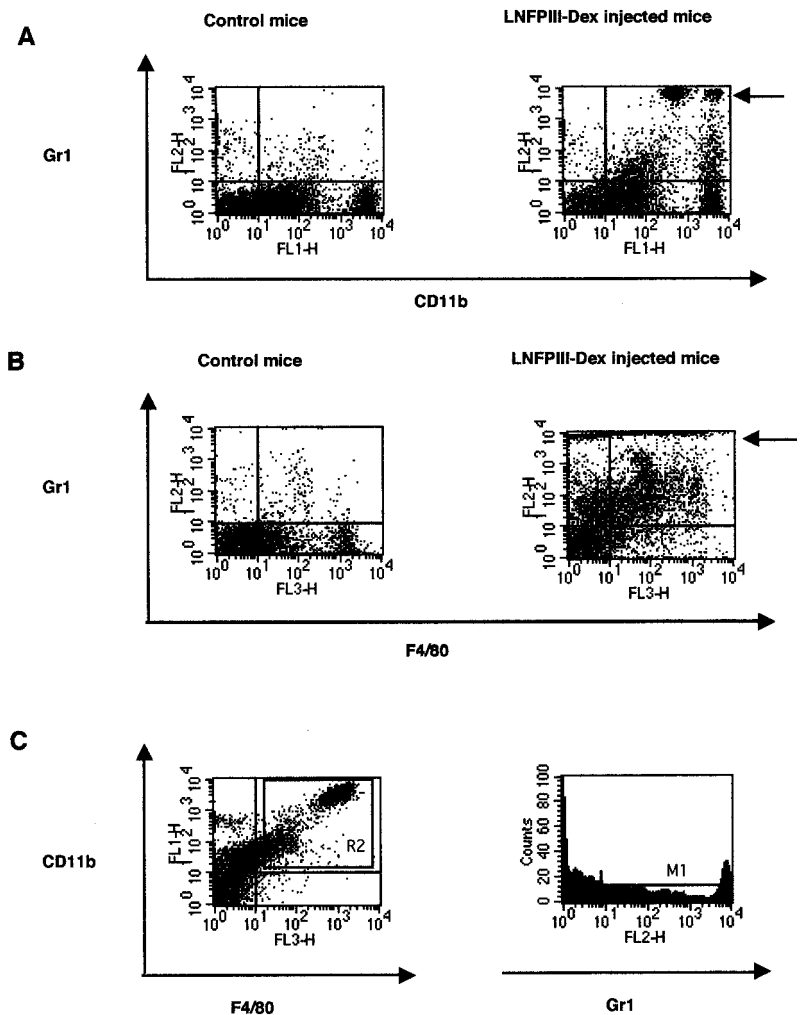


FIGURE 1. LNFPIII-dex expands Gr1⁺ cells in the PC of BALB/c mice. Twenty hours after being injected with dextran or LNFPIII-dex, peritoneal cells were harvested by saline lavage. *A*, Cells were stained with Abs to CD11b and Gr1. *B*, Cells were stained with Abs to F4/80 and Gr1. *C*, In the *left panel*, CD11b/F4/80 double-positive cells were gated (R2); the *right panel* shows a histogram of the % Gr1⁺ cells of the R2-gated population. The plots shown in this figure are representative of seven different experiments. Arrow indicates presence of Gr1^{high} cells.

splenocytes were stained with the proliferation marker CFSE before stimulation with anti-CD3 and anti-CD28 Abs (26). CFSE-labeled splenocytes were incubated in 48-well plates coated with anti-CD3/CD28 mAbs for 3 h, then mixed with varying numbers of PECs from uninjected, dex-injected, or LNFPIII-dex-injected mice. In the current study, we gated CD4⁺ T cells and analyzed their proliferation. Fig. 2 shows data from a representative experiment where PECs from mice injected with LNFPIII-dex, at ratios of 1:4 and 1:8 (PECs to splenocytes), significantly suppressed CD4⁺ T cell proliferation compared with PECs from control mice at the same ratios.

Suppression caused by PECs from LNFPIII-dex-injected mice is NO-dependent

Activated macrophages often suppress T cell activity via NO production. Measurement of NO production in supernatants of cocul-

tured PECs and naive splenocytes is shown in Table II. In all experiments, at all ratios of PECs to splenocytes, slightly higher nitrite levels were detected in coculture supernatants containing PECs from LNFPIII-injected mice. Neither PECs nor splenocytes alone generated NO (data not shown).

To confirm that NO was involved in the suppression of naive splenocytes, we added L-nMMA, an inhibitor of inducible NO synthase to cocultures. In preliminary experiments, we checked the effects of not only L-nMMA but also of a superoxide dismutase mimetic (MnTBAP). MnTBAP did not reverse immune suppression at any concentrations tested (data not shown). However, addition of L-nMMA to cocultures completely restored the ability of CD4 T cells to proliferate in response to stimulation with anti-CD3/CD28 mAbs at all ratios of suppressors to responders examined (Fig. 3A). NO production in supernatants obtained from the same cocultures is shown in Fig. 3B. The concentration of NO was dependent on the numbers of PECs in coculture with naive splenocytes. L-nMMA decreased NO in cell cultures to background levels.

An IFN- γ -dependent mechanism of inhibition of T cell proliferation in vitro

NS macrophages suppress via IFN- γ - or IL-4-dependent mechanisms. In addition, NO production by suppressor cells has been linked with an IFN- γ -dependent mechanism (5). To further define the suppressive mechanism used by LNFPIII-dex PECs, we performed coculture experiments with neutralizing Abs to IFN- γ or IL-4.

Table I. Percentage of Gr1⁺ cells between groups of control, dextran-, and LNFPIII-dex-injected mice^a

	% Gr1 ⁺ /CD11b ⁺	% Gr1 ⁺ of CD11b ⁺ /F4/80 ⁺
Uninjected	4.98 ± 0.48	18.48 ± 1.83
Dextran	4.03 ± 0.47	18.39 ± 1.88
LNFPIII-dex	36.47 ± 2.27*	75.55 ± 2.10*

^a PECs obtained from control, dextran-, or LNFPIII-dex-injected mice were stained with Abs against Gr1/CD11b and F4/80. The results shown are representative of several different experiments. *, $p < 0.001$ between control (uninjected and dex-injected) and LNFPIII-dex-injected mice.

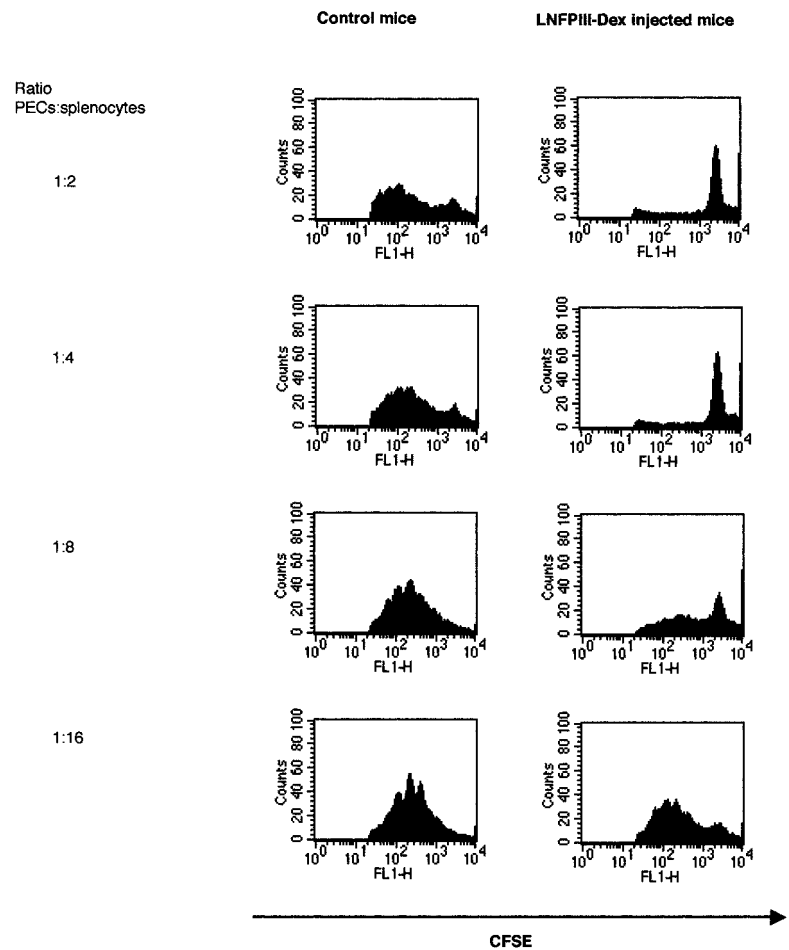


FIGURE 2. PECs from LNFPIII-dex-injected mice suppress proliferation of naive CD4⁺ T cells. Naive splenocytes were labeled with CFSE dye (see *Materials and Methods*) then plated (0.5×10^6 /well) onto 48-well plates coated with Abs to CD3 (1 μ g/ml) and CD28 (5 μ g/ml). Three hours later, PECs from control or LNFPIII-dex-injected mice were added at the indicated ratios to the wells containing the splenocytes. Cells were harvested after 72 h of coculture and stained for CD4. During FACS analysis, gating was on the CD4⁺ population of cells. Data are representative of five separate experiments.

Fig. 4 demonstrates that addition of anti-IFN- γ Abs to cocultures completely restored the proliferative activity of naive CD4⁺ T cells activated with anti-CD3/CD28 mAbs. In contrast, CD4⁺ T cells in cocultures containing anti-IL-4, anti-IL-10 (data not shown), or isotype control Abs did not restore proliferation, demonstrating that LNFPIII-dex expanded suppressor macrophages were functioning via an IFN- γ , but not an IL-4- or IL-10-dependent mechanism. NO production of cocultured PECs and splenocytes was reduced to 2.5 μ M at all concentrations of PECs in wells where anti-IFN- γ was added. Thus, IFN- γ is a critical factor for development of LNFPIII-dex-induced PEC suppressor activity.

Gr1^{high}-positive cells are responsible for the suppression of anti-CD3/CD28-induced T cell proliferation

Previous studies have shown that suppression of T cell proliferation is linked to the presence of Gr1⁺ cells, which also express

Table II. NO production (μ M) by cocultures of CD3/CD28-stimulated naive splenocytes and PECs^a

Ratio PECs: Splenocytes	Uninjected	Dex-Injected	LNFPIII-Dex-Injected
1:2	16.52 \pm 0.39	18.58 \pm 1.04	22.55 \pm 0.67*†
1:4	11.32 \pm 0.40	11.62 \pm 0.63	14.52 \pm 0.65*†
1:8	6.00 \pm 0.41	8.68 \pm 0.85	10.12 \pm 0.35*†
1:16	3.47 \pm 0.38	4.53 \pm 0.29	7.37 \pm 0.61*†

^a Naive splenocytes were preincubated for 3 h with anti-CD3/CD28 mAbs, then PECs from control (uninjected or dex-injected) and LNFPIII-dex-injected mice were added at the above ratios. After 72 h of coculture, supernatants were harvested and NO products were assayed. *, $p < 0.05$ between uninjected and LNFPIII-dex-injected mice; † $p < 0.05$ between DEX-injected and LNFPIII-dex-injected mice.

CD11b and/or F4/80 (13, 14). To identify whether suppression was due to Gr1⁺ macrophages, we used positively selected Gr1⁺/F4/80 cells. In these experiments, PECs were separated into Gr1^{+/−} and F4/80^{+/−} populations as described in *Materials and Methods*. FACS staining (Fig. 5A) confirmed the efficiency of the selection. The Gr1⁺ subpopulation contained 99.7% positive cells (data not shown). The ability of Gr1⁺/F4/80⁺ and the various depleted subpopulations to suppress naive splenocytes was tested in coculture experiments. The population that was depleted of F4/80⁺/Gr1^{high} cells still contained Gr1⁺ cells, although the overall fluorescence intensity was low (Fig. 5A, right panel). In coculture experiments we found that the Gr1^{high}, F4/80⁺ purified subpopulation was responsible for suppression. The representative pattern in Fig. 5B shows that double-positive cells blocked T cell proliferation even at a ratio of 1:16 (PECs to splenocytes). In contrast, the PEC population that contained Gr1^{low}/F4/80[−] cells did not inhibit splenocyte proliferation, nor did the F4/80⁺/Gr1^{low} population.

To confirm the role for F4/80⁺ cells in suppression, we isolated F4/80⁺ cells using anti-FITC microbeads. Gr1⁺ cells were isolated from this same population of F4/80⁺ cells by anti-PE microbeads and MACS column separation. It was interesting to note that following purification, all F4/80⁺ cells expressed the Gr1 marker, yet in contrast, not all Gr1⁺ cells express the F4/80 marker. Similar to what was observed with total PECs from LNFPIII-dex-injected mice, suppression by purified Gr1⁺ cells was also IFN- γ -dependent, as anti-IFN- γ mAbs completely abrogated suppression at all ratios of suppressor/responder (Fig. 5C). Supernatants from cocultures of Gr1[−]/F4/80[−] PECs with splenocytes contained small amounts of NO products, as opposed to cocultures of splenocytes and Gr1⁺/F4/80⁺ PECs, which contained high

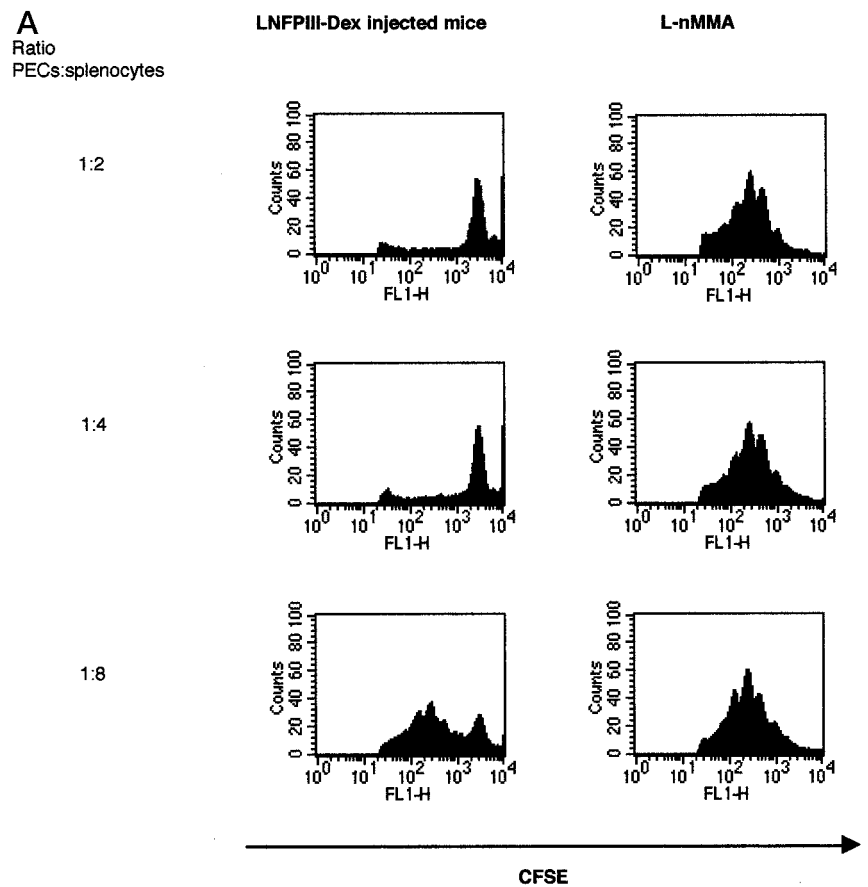
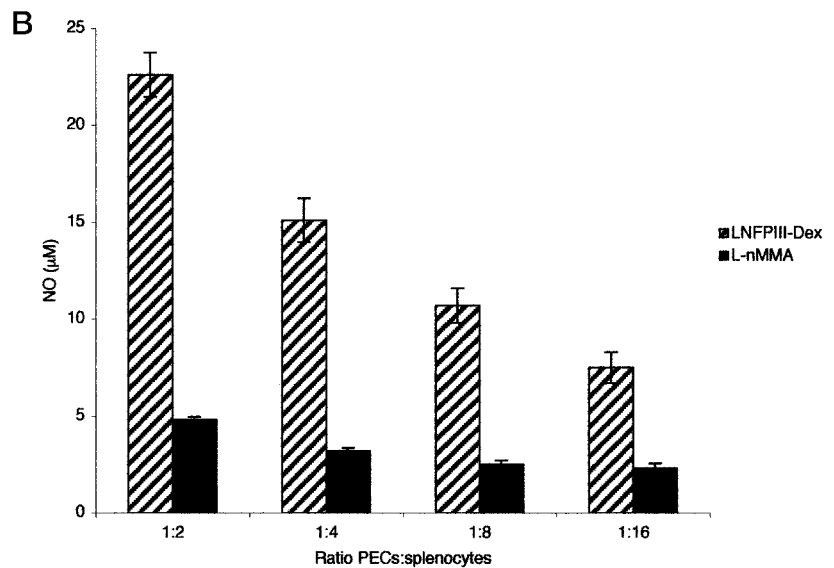


FIGURE 3. NO production is associated with suppression of splenocyte proliferation by PECs from LNFPIII-dex-injected mice. *A*, Inducible NO synthase inhibition by L-nMMA abrogates PEC suppression of naive splenocytes. *Left panel*, CFSE fluorescence intensity in cultures without L-nMMA. *Right panel*, Cultures with L-nMMA (0.5 mM). *B*, Levels of NO produced in cocultures with or without L-nMMA.



amounts of NO (data not shown). Thus, F4/80⁺/Gr1⁺ double-positive macrophages are responsible for the observed suppressive activity of PECs from LNFPIII-dex-injected mice.

Mechanism of PEC suppression is T cell-independent

In normal mice, resident PECs include macrophages, neutrophils, and NK cells, as well as T and B cells. We injected LNFPIII-dex or dextran into SCID mice to determine whether the LNFPIII-dex-induced accumulation of Gr1⁺ macrophages was dependent on the presence of T cells in the PEC population. Table III compares the Gr1⁺ cells of the CD11b⁺/F4/80⁺ population in both SCID and BALB/c mice 20 h after LNFPIII-dex or dex injection. In LNFPIII-

dex-injected SCID mice, 92.5 ± 2.5% of the CD11b⁺/F4/80⁺ cells were Gr1⁺ vs 75.6 ± 2.1% in BALB/c, although absolute numbers of PECs from SCID mice were substantially less than those from BALB/c (data not shown). The greater percentage of Gr1⁺ cells seen in SCID mice compared with BALB/c mice may be due to immunocompensatory mechanisms in SCID mice.

The PECs from SCID mice injected with LNFPIII-dex were functional suppressors, as shown in Fig. 6. The degree of suppression was higher than in wild-type mice, even when the ratio of PECs to splenocytes was 1:16, and inhibition was also NO dependent, as addition of L-nMMA to cocultures completely restored proliferation of CD4⁺ T cells at all ratios of suppressor-responder.

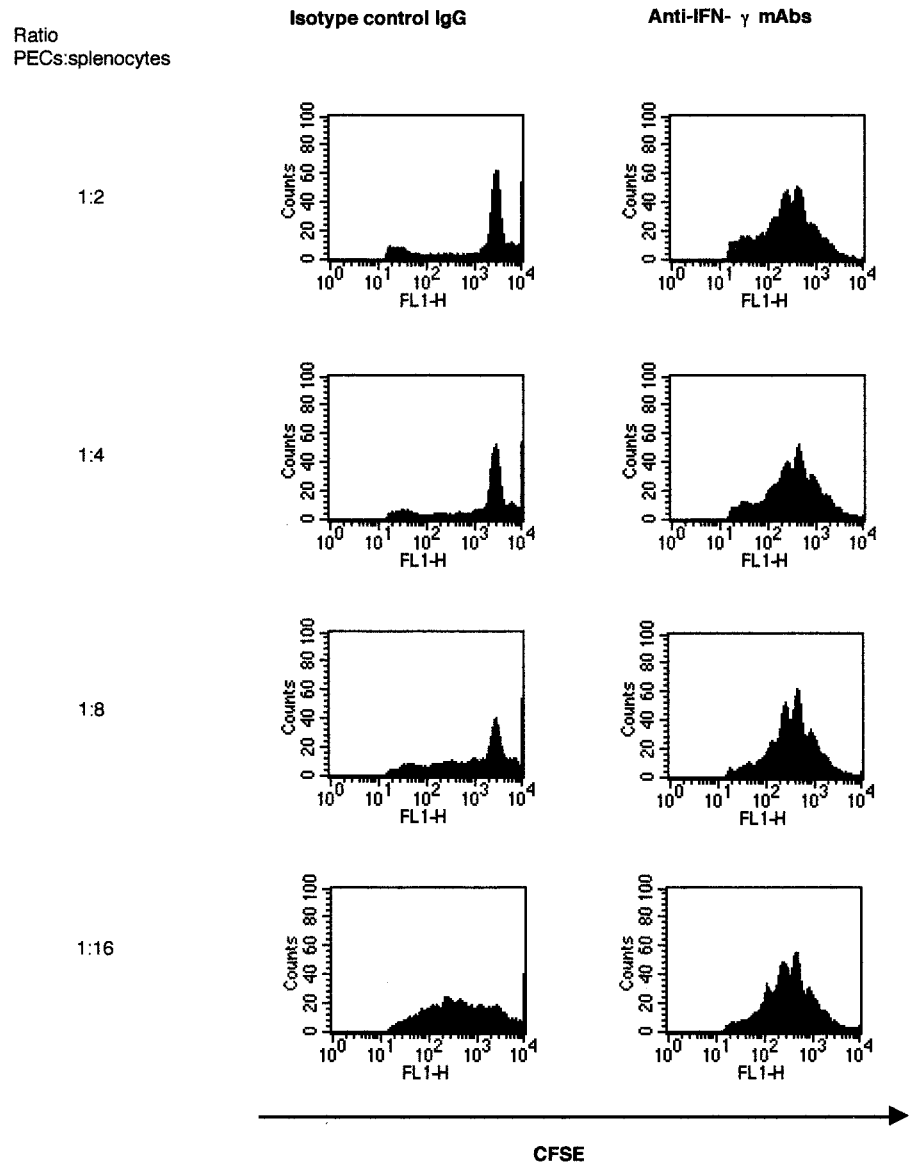


FIGURE 4. IFN- γ is required for PEC suppression of T cell proliferation. Histograms show CFSE fluorescence intensity in T cells from cocultures containing isotype control Abs (*left*) or Abs to IFN- γ (10 μ g/ml, *right*). Data are representative of three separate experiments.

For both strains of mice, the amount of NO products present was found to be dependent on the number of PECs in culture, although the difference in the amount of NO products between control and SCID mice was not significant (data not shown). This may be due to the existence of a “ceiling” concentration of NO products.

Discussion

The ability of innate responses to influence or direct subsequent adaptive responses is an area of intense research activity. In the majority of cases, the initial pro-inflammatory response is followed by a Th1-type adaptive response. In contrast, helminth infection, which may be characterized by an initial pro-inflammatory Th1-type response, quickly switches to a polarized Th2 response along with the production of the anti-inflammatory mediator, IL-10 (4). In situations where intense pro-inflammatory responses are initiated, the ability to abrogate or down-regulate these responses is critical for survival. In this regard, there has been considerable recent interest in a population of cells termed “natural suppressors”, which have been shown to inhibit T and/or B cell proliferation. NS cells have been phenotypically defined as Gr1⁺ and CD11b⁺ cells, with one report demonstrating that they also express the murine macrophage marker F4/80 on NS cells (27). The

peritoneal suppressor macrophages described in this study express each of these surface molecules, similar to suppressor macrophages reported earlier (19, 28). Gr1 is expressed on granulocytes, monocytes, and immature myeloid precursors in bone marrow, and at low levels on splenocytes of normal mice (7, 17, 28). CD11b (Mac1) is a leukocyte adhesion molecule found on the surface of monocytes/macrophages, granulocytes, NK cells, and B-1 cells (10). F4/80 is a 160-kDa glycoprotein (29) specific for murine macrophages (30, 31).

Because expansion of NS cell populations in helminth-infected or tumor-bearing mice has been reported to take days to weeks, this phenomenon has not been considered an innate response. However, in contrast to these studies, we demonstrated that injection of LNFPIII-dex significantly expanded the peritoneal suppressor macrophage population within 20 h. This observation, coupled with the results from a study showing that suppressor macrophage populations could be increased within 48 h by injection of superantigen, suggests that expansion of this cell type may occur through an innate immune response triggered by ligation of pattern recognition receptors (14).

Although all suppressor macrophages described to date have similar/identical surface markers, two distinct subpopulations of

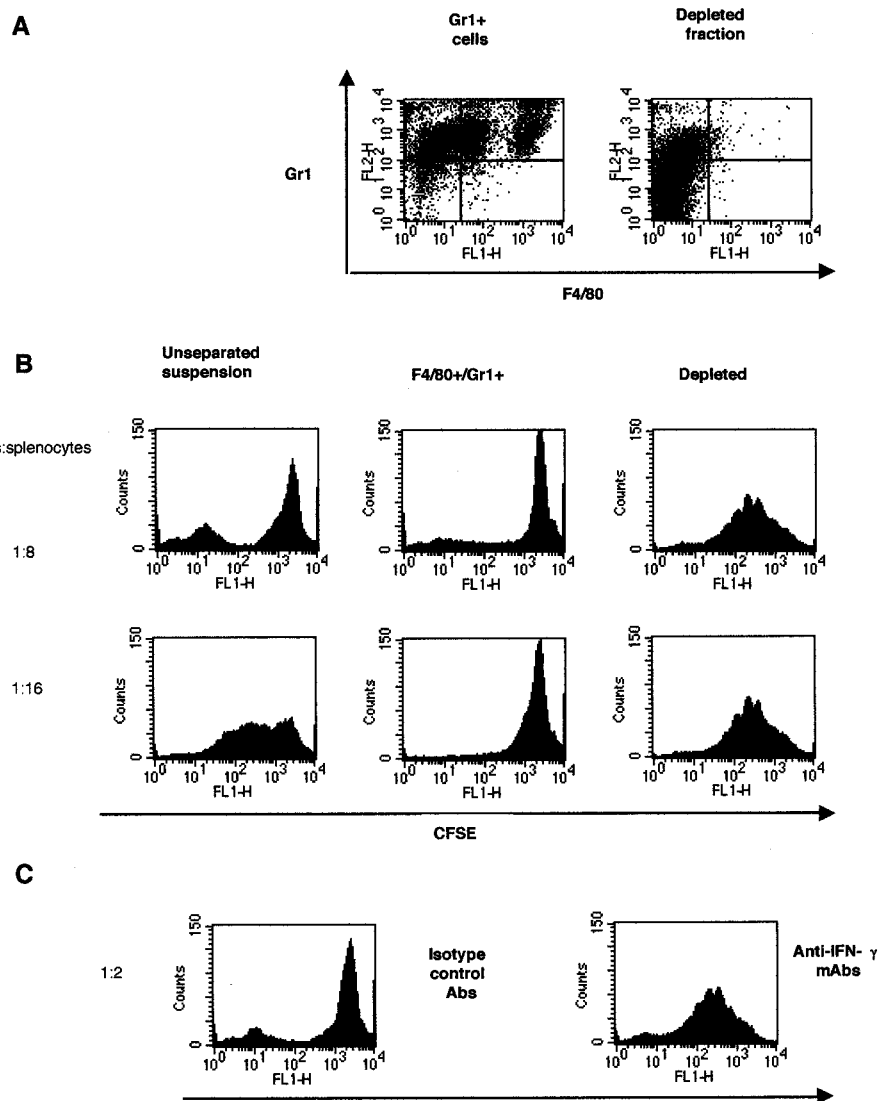


FIGURE 5. The Gr1⁺ subpopulation of F4/80⁺ PECs is responsible for T cell suppression in an IFN- γ -dependent mechanism. *A*, Gr1⁺/F4/80⁺ and Gr1^{low}/F4/80⁻ PEC populations, shown in the *left* and *right panels*, respectively, were purified as described in *Materials and Methods*. *B*, CFSE fluorescence intensity of T cells cocultured with total PECs (*left*), F4/80⁺/Gr1⁺ PECs (*center*), or F4/80⁻/Gr1^{low} PECs (*right*). *C*, Suppression of cocultured T cells by F4/80⁺/Gr1⁺ PECs is dependent on IFN- γ . Data are representative of three separate experiments.

suppressors have been characterized. CA suppressor macrophages are NO- and IFN- γ -dependent (13, 14), whereas AA macrophages are IL-4-dependent (11, 16, 18, 19, 21, 27, 28). CA macrophages have been reported in mice injected with superantigens, tumors, viruses, cyclophosphamide, and other Ags (13–15, 32). The Gr1⁺ suppressor macrophage population we described in this study falls into the CA category. The cell population expanded by injection of LNFPIII-dex-suppressed T cell proliferation via an NO- and IFN- γ -dependent mechanism, as demonstrated by addition of L-nMMA or anti-IFN- γ mAbs, respectively. Furthermore, although addition of anti-IFN- γ mAbs completely abrogated suppression, addition of Abs to IL-4 had no effect. That LNFPIII-dex expands peritoneal cells, which suppress naive T cells in an NO- and IFN- γ -dependent mechanism, is nearly identical with that reported for the structur-

ally similar schistosome oligosaccharide, LNnT-dex.³ The results with both glycoconjugates are consistent with several studies including that of Angulo and coworkers (15), who showed that Gr1⁺/CD11b⁺/CD31⁺ cells from cyclophosphamide-treated mice inhibited mitogen-induced splenic T cell responses through an NO-dependent mechanism, which was IFN- γ -dependent. The study of Brooks and Hoskin (32) also demonstrated that CD11b⁺ cyclophosphamide-treated spleen cells were dependent on the presence of IFN- γ for expression of inhibitory activity. Additionally, IFN- γ has been suggested as an inducible NO synthase inducer in NS cells (5). Although the study reported here and those of Angulo et al. (5) and Brooks and Hoskin (32) demonstrated that the suppressive mechanism was NO- and IFN- γ -dependent, other signals that we did not examine, such as TNF- α , CD40, and LPS, may be required for successful NO production (5, 15).

Macrophages respond to Ag exposure by producing IL-12 and TNF- α , activating CD4, CD8, and NK cells for IFN- γ release (33–37). IFN- γ has been shown to stimulate NO production by macrophages (38–40) and induce apoptosis of activated CD4 T cells and macrophages in vitro during bacillus Calmette-Guérin infection, a mechanism that involves NO (41). It was previously reported that NO, or products of the L-arginine pathway of reactive nitrogen intermediates, mediated bone marrow-derived NS activity (5, 15). The data presented here are consistent with these earlier

Table III. Percent Gr1⁺ of CD11b⁺/F4/80⁺ cells from BALB/c and SCID mice^a

	Control	Dextran	LNFPIII-Dex
SCID	25.24 ± 2.62	21.14 ± 3.69	92.54 ± 2.49
BALB/c	18.48 ± 1.83	18.39 ± 1.88	75.55 ± 2.10*

^a PECs obtained from control, dextran, or LNFPIII-Dex-injected mice were stained with Abs against Gr1/CD11b and F4/80. *, *p* < 0.05 between SCID and BALB/c.

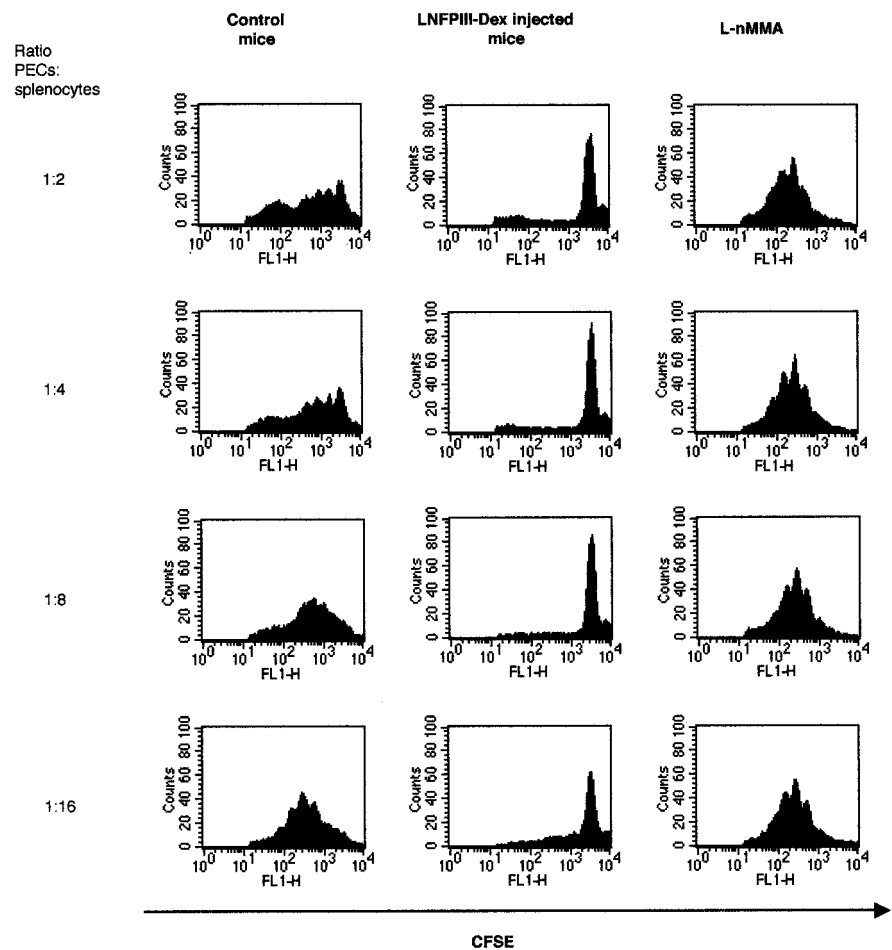


FIGURE 6. LNFPIII-dex drives an NO-dependent suppressor PEC population in SCID mice. Histograms show CFSE fluorescence intensity in T cells cocultured with PECs from control mice (*left*), LNFPIII-dex-injected mice (*center*), and LNFPIII-dex-injected mice in the presence of L-nMMA (*right*). Data shown are representative of three separate experiments.

studies, as we demonstrated that NS activity was greatly inhibited by L-nMMA, a competitive inhibitor of arginine-dependent NO synthase (42, 43). The mechanism by which NO might act in this manner is still unclear. Although our data show an important role of NO in Gr1⁺ PEC suppression of T cell proliferation, they do not formally demonstrate this agent as the ultimate suppressor molecule. The involvement of NO as a suppressive factor for proliferative responses of lymphocytes has already been shown for peritoneal macrophages in another parasite-infected mouse model (44).

The rapid expansion of suppressor macrophages observed here, following injection of LNFPIII-dex, has not previously been observed. Twenty hours after injection of LNFPIII-dex, the percentage of Gr1⁺ cells as a subpopulation of F4/80⁺/CD11b⁺ PECs was expanded >4-fold, to 75%, compared with 18% in uninjected or dex-injected mice. Using methods similar to those reported by Kusmartsev (13), we demonstrated that these cells were functional suppressors, as they inhibited the proliferation of anti-CD3/CD28-stimulated naive T cells *in vitro* at ratios of 1:4 and 1:8 (PECs-splenocytes), whereas PECs from control mice did not. Similar data were obtained using the structurally related glycoconjugate LNT-dex.³ These data are also consistent with the results of Cauley and coworkers (14), who showed that Gr1⁺ splenocytes from mice given soluble egg Ag completely inhibited the proliferative response of CD4⁺ T cells at a ratio of 1:8. This same study also demonstrated that Gr1⁺ cells from control mice had the ability to suppress naive T cells when they were cocultured at a ratio of 1:2 (PECs-T cells). The suppressor population described in our study suppressed via NO and was IFN- γ -dependent and IL-4-independent. Thus, in comparison to the studies that generated IL-4-dependent, AA suppressor populations including those examining

suppressor cells from filaria-harboring mice (11), injection of LNFPIII-dex led to expansion of CA, IFN- γ -dependent suppressors. A simple explanation for the presence of CA NS cells following injection of LNFPIII-dex, vs AA PECs following implantation of filarial parasites, may be because LNFPIII is not found on filaria (45). Furthermore, LNFPIII is found on a subset of tumors in addition to schistosomes (23, 25, 46–48). The difference may also be due to something as simple as differences between injection of the glycoconjugate vs implantation of several living metazoan parasites.

In addition to demonstrating that the mechanism of suppression was NO- and IFN- γ -dependent, we also demonstrated that Gr1⁺/F4/80⁺ PECs depleted of Gr1⁺ cells were no longer able to suppress naive T cells, functionally showing that the Gr1⁺ macrophages are the suppressors and that Gr1^{low}/F4/80⁻ cells are not. Injection of LNFPIII-dex gave rise to two populations of Gr1⁺ cells, which we termed high and low. This observation on two Gr1⁺ populations is similar to that reported for Gr1⁺ populations described in the spleens of superantigen-treated mice, which were shown to express high levels of CD11b and LFA-1 (14). Salvatory and coworkers (8) also found two populations of Gr1⁺ cells in splenocytes from naive and tumor-bearing mice. In the tumor-bearing host, the majority of Gr1⁺ cells stained brightly for Gr1 (Gr1^{high}), with very few Gr1^{low} cells. In our study, depletion of either Gr1^{high} or F4/80⁺/Gr1⁺ cells from PEC populations of LNFPIII-dex-injected mice eliminated the ability of the PECs to suppress naive T cell proliferation.

The last experiments we performed on suppressor macrophages were to determine whether T cells were required for their expansion and/or function as suppressors. Considering the rapid time

frame in which the suppressor population expanded following LNFPIII-dex injection, we felt it was unlikely that these cells required other cell populations, and that they likely directly interacted with the LNFPIII-dex conjugate, leading to up-regulation of Gr1. We did inject LNFPIII-dex into SCID mice and found similar kinetics of the PEC suppressor population, showing that this phase was T cell-independent. Furthermore, Gr1⁺ suppressor cells from SCID mice were functional suppressors, and the mechanism of suppression was also NO-dependent. The findings on NO production are similar to earlier studies demonstrating that macrophages from SCID spleens produced large amounts of NO when incubated with heat-killed *Listeria monocytogenes*, but only in the presence of IFN- γ (49). This T cell-independent response relied on the presence of both macrophages and NK cells, and was modulated by the addition of mAbs against F4/80 (2). In the current study we demonstrated that increased numbers of Gr1⁺/F4/80⁺ cells were accompanied by strong suppression and NO production during coculture of PECs and naive splenocytes, and the effects of suppression and NO production were abrogated by addition of an inhibitor of NO synthase (L-nMMA).

Taken together, injection of the *S. mansoni* expressed immunoregulatory oligosaccharide as the neo-glycoconjugate LNFPIII-dex rapidly expanded a peritoneal suppressor macrophage population that phenotypically and mechanistically resembles the CA suppressor macrophages defined in viral, tumor-, and superantigen-exposed murine systems (12–15). The kinetics of expansion of the suppressor macrophages reported here are the most rapid observed to date, and suggest an innate response. Indeed, we have shown using the structurally related, nonfucosylated homologue LNT-dex conjugate, that suppressor macrophages are expanded as rapidly as 2 h postinjection.³ How the generation of such a suppressor population might alter immune responses during schistosome infection is currently being investigated. One can speculate that suppressor cells, if they are actually expanded during infection, would dampen cell-mediated responses, and possibly help bias the ensuing response to Th2-type. The latter point is based on the observation that suppressor PECs obtained from mice injected with LNT-dex produce lower levels of Th1 cytokines and enhanced levels of IL-10 and TGF- β .³ In this regard, we have observed the appearance of Gr1⁺ suppressors in the PCs of schistosome-infected mice at 6 wk postinfection, which is the earliest we have looked (data not shown). Experiments where this population is eliminated from infected animals should help define a role for suppressor macrophages during natural infection.

Acknowledgments

We thank Drs. Akram Da'dara and Luis Terrazas, and Paul Thomas for their helpful commentary.

References

- Medzhitov, R., and C. Janeway. 2000. Innate immune recognition: mechanisms and pathways. *Immunol. Rev.* 173:89.
- Warschkau, H., and A. F. Kiderlen. 1999. A monoclonal antibody directed against the murine macrophage surface molecule F4/80 modulates natural immune response to *Listeria monocytogenes*. *J. Immunol.* 163:3409.
- Goerd, S., and C. E. Orfanos. 1999. Other functions, other genes: alternative activation of antigen-presenting cells. *Immunity* 10:137.
- Flores Villanueva, P. O., T. S. Harris, D. E. Ricklan, and M. J. Stadecker. 1994. Macrophages from schistosomal egg granulomas induce unresponsiveness in specific cloned Th-1 lymphocytes in vitro and down-regulate schistosomal granulomatous disease in vivo. *J. Immunol.* 152:1847.
- Angulo, I., R. Rodriguez, B. Garcia, M. Medina, J. Navarro, and J. L. Subiza. 1995. Involvement of nitric oxide in bone marrow-derived natural suppressor activity: its dependence on IFN- γ . *J. Immunol.* 155:15.
- Moore, S. C., M. A. Shaw, and L. S. Soderberg. 1992. Transforming growth factor- β is the major mediator of natural suppressor cells derived from normal bone marrow. *J. Leukocyte Biol.* 52:596.
- Maruyama, S., M. Minagawa, T. Shimizu, H. Oya, S. Yamamoto, N. Musha, W. Abo, A. Weerasinghe, K. Hatakeyama, and T. Abo. 1999. Administration of glucocorticoids markedly increases the numbers of granulocytes and extrathymic T cells in the bone marrow. *Cell. Immunol.* 194:28.
- Salvadori, S., G. Martinelli, and K. Zier. 2000. Resection of solid tumors reverses T cell defects and restores protective immunity. *J. Immunol.* 164:2214.
- MacDonald, A. S., R. M. Maizels, R. A. Lawrence, I. Dransfield, and J. E. Allen. 1998. Requirement for in vivo production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. [Published erratum appears in 1998 *J. Immunol.* 160:4124.] *J. Immunol.* 160:1304.
- MacDonald, A. S., P. Loke, and J. E. Allen. 1999. Suppressive antigen-presenting cells in helminth infection. *Pathobiology* 67:265.
- Loke, P., A. S. MacDonald, A. Robb, R. M. Maizels, and J. E. Allen. 2000. Alternatively activated macrophages induced by nematode infection inhibit proliferation via cell-to-cell contact. *Eur. J. Immunol.* 30:2669.
- Young, M. R., M. A. Wright, J. P. Matthews, I. Malik, and M. Prechel. 1996. Suppression of T cell proliferation by tumor-induced granulocyte-macrophage progenitor cells producing transforming growth factor- β and nitric oxide. *J. Immunol.* 156:1916.
- Kusmartsev, S. A., Y. Li, and S. H. Chen. 2000. Gr-1⁺ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J. Immunol.* 165:779.
- Cauley, L. S., E. E. Miller, M. Yen, and S. L. Swain. 2000. Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and IFN- γ . *J. Immunol.* 165:6056.
- Angulo, I., F. G. de las Heras, J. F. Garcia-Bustos, D. Gargallo, M. A. Munoz-Fernandez, and M. Fresno. 2000. Nitric oxide-producing CD11b⁺Ly-6G(Gr-1)⁺CD31(ER-MP12)⁺ cells in the spleen of cyclophosphamide-treated mice: implications for T-cell responses in immunosuppressed mice. *Blood* 95:212.
- Stein, M., S. Keshav, N. Harris, and S. Gordon. 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J. Exp. Med.* 176:287.
- Bronte, V., M. Wang, W. W. Overwijk, D. R. Surman, F. Pericle, S. A. Rosenberg, and N. P. Restifo. 1998. Apoptotic death of CD8⁺ T lymphocytes after immunization: induction of a suppressive population of Mac-1⁺/Gr-1⁺ cells. *J. Immunol.* 161:5313.
- Bronte, V., D. B. Chappell, E. Apolloni, A. Cabrelle, M. Wang, P. Hwu, and N. P. Restifo. 1999. Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8⁺ T cell responses by dysregulating antigen-presenting cell maturation. *J. Immunol.* 162:5728.
- Apolloni, E., V. Bronte, A. Mazzoni, P. Serafini, A. Cabrelle, D. M. Segal, H. A. Young, and P. Zanovello. 2000. Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. *J. Immunol.* 165:6723.
- Allen, J. E., R. A. Lawrence, and R. M. Maizels. 1996. APC from mice harbouring the filarial nematode, *Brugia malayi*, prevent cellular proliferation but not cytokine production. *Int. Immunol.* 8:143.
- Goerd, S., O. Politz, K. Schledzewski, R. Birk, A. Gratchev, P. Guillot, N. Hakiy, C. D. Klemke, E. Dippel, V. Kodelja, and C. E. Orfanos. 1999. Alternative versus classical activation of macrophages. *Pathobiology* 67:222.
- Okano, M., A. Sato, F. Brombacher, and D. Harn. 2001. Lacto-N-fucopentaose III found on *Schistosoma mansoni* egg antigens functions as adjuvant for proteins by inducing Th2-type response. *J. Immunol.* 167:442.
- Velupillai, P., and D. A. Harn. 1994. Oligosaccharide-specific induction of interleukin 10 production by B220⁺ cells from schistosome-infected mice: a mechanism for regulation of CD4⁺ T-cell subsets [see comments]. *Proc. Natl. Acad. Sci. USA* 91:18.
- Velupillai, P., W. E. Secor, A. M. Harauf, and D. A. Harn. 1997. B-1 cell (CD5⁺B220⁺) outgrowth in murine schistosomiasis is genetically restricted and is largely due to activation by polylectosamine sugars. *J. Immunol.* 158:338.
- Velupillai, P., E. A. dos Reis, M. G. dos Reis, and D. A. Harn. 2000. Lewis(x)-containing oligosaccharide attenuates schistosome egg antigen-induced immune depression in human schistosomiasis. *Hum. Immunol.* 61:225.
- Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171:131.
- Loke, P., A. S. MacDonald, and J. E. Allen. 2000. Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive CD4⁺ T cells. *Eur. J. Immunol.* 30:1127.
- Bronte, V., E. Apolloni, A. Cabrelle, R. Ronca, P. Serafini, P. Zamboni, N. P. Restifo, and P. Zanovello. 2000. Identification of a CD11b⁺/Gr-1⁺/CD31⁺ myeloid progenitor capable of activating or suppressing CD8⁺ T cells. *Blood* 96:3838.
- McKnight, A. J., A. J. Macfarlane, P. Dri, L. Turley, A. C. Willis, and S. Gordon. 1996. Molecular cloning of F4/80, a murine macrophage-restricted cell surface glycoprotein with homology to the G-protein-linked transmembrane 7 hormone receptor family. *J. Biol. Chem.* 271:486.
- Austyn, J. M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* 11:805.
- Hirsch, S., J. M. Austyn, and S. Gordon. 1981. Expression of the macrophage-specific antigen F4/80 during differentiation of mouse bone marrow cells in culture. *J. Exp. Med.* 154:713.
- Brooks, J. C., and D. W. Hoskin. 1994. The inhibitory effect of cyclophosphamide-induced MAC-1⁺ natural suppressor cells on IL-2 and IL-4 utilization in MLR. *Transplantation* 58:1096.
- Tripp, C. S., S. F. Wolf, and E. R. Unanue. 1993. Interleukin 12 and tumor necrosis factor α are costimulators of interferon γ production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist. *Proc. Natl. Acad. Sci. USA* 90:3725.

34. Flesch, I. E., J. H. Hess, S. Huang, M. Aguet, J. Rothe, H. Bluethmann, and S. H. Kaufmann. 1995. Early interleukin 12 production by macrophages in response to mycobacterial infection depends on interferon γ and tumor necrosis factor α . *J. Exp. Med.* 181:1615.
35. Anguita, J., D. H. Persing, M. Rincon, S. W. Barthold, and E. Fikrig. 1996. Effect of anti-interleukin 12 treatment on murine lyme borreliosis. *J. Clin. Invest.* 97:1028.
36. Derrico, C. A., and K. J. Goodrum. 1996. Interleukin-12 and tumor necrosis factor α mediate innate production of γ interferon by group B streptococcus-treated splenocytes of severe combined immunodeficiency mice. *Infect. Immun.* 64:1314.
37. Desmedt, M., P. Rotiers, H. Doms, W. Fiers, and J. Grooten. 1998. Macrophages induce cellular immunity by activating Th1 cell responses and suppressing Th2 cell responses. *J. Immunol.* 160:5300.
38. Alleva, D. G., C. J. Burger, and K. D. Elgert. 1994. Tumour growth causes suppression of autoreactive T-cell proliferation by disrupting macrophage responsiveness to interferon- γ . *Scand J. Immunol.* 39:31.
39. Young, M. R., G. McCloskey, M. A. Wright, and A. S. Pak. 1994. Increasing infiltration and activation of CD8⁺ tumor-infiltrating lymphocytes after eliminating immune suppressive granulocyte/macrophage progenitor cells with low doses of interferon γ plus tumor necrosis factor α . *Cancer Immunol. Immunother.* 38:9.
40. Willenborg, D. O., M. A. Staykova, and W. B. Cowden. 1999. Our shifting understanding of the role of nitric oxide in autoimmune encephalomyelitis: a review. *J. Neuroimmunol.* 100:21.
41. Dalton, D. K., L. Haynes, C. Q. Chu, S. L. Swain, and S. Wittmer. 2000. Interferon γ eliminates responding CD4 T cells during mycobacterial infection by inducing apoptosis of activated CD4 T cells. *J. Exp. Med.* 192:117.
42. Moncada, S., and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* 329:2002.
43. Hibbs, J. B., Jr., Z. Vavrin, and R. R. Taintor. 1987. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.* 138:550.
44. Albina, J. E., J. A. Abate, and W. L. Henry, Jr. 1991. Nitric oxide production is required for murine resident peritoneal macrophages to suppress mitogen-stimulated T cell proliferation: role of IFN- γ in the induction of the nitric oxide-synthesizing pathway. *J. Immunol.* 147:144.
45. Nyame, A., R. Debose-Boyd, T. Long, V. Tsang, and R. Cummings. 1998. Expression of Lex antigen in *Schistosoma japonicum* and *S. haematobium* and immune responses to Lex in infected animals: lack of Lex expression in other trematodes and nematodes. *Glycobiology* 8:615.
46. Sittel, C., H. E. Eckel, M. Damm, E. von Pritzbuer, and H. M. Kvasnicka. 2000. Ki-67 (MIB1), p53, and Lewis-X (LeuM1) as prognostic factors of recurrence in T1 and T2 laryngeal carcinoma. *Laryngoscope* 110:1012.
47. Ko, A. I., U. C. Drager, and D. A. Harn. 1990. A *Schistosoma mansoni* epitope recognized by a protective monoclonal antibody is identical to the stage-specific embryonic antigen 1. *Proc. Natl. Acad. Sci. USA* 87:4159.
48. Jacobs, W., A. Deelder, and E. Van Marck. 1999. *Schistosomal* granuloma modulation. II. Specific immunogenic carbohydrates can modulate schistosome-egg-antigen-induced hepatic granuloma formation. *Parasitol. Res.* 85:14.
49. Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue. 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation: its role in resistance to *Listeria monocytogenes*. *J. Immunol.* 150:888.