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In Vitro Simulation of Immunosuppression Caused by *Trypanosoma brucei*: Active Involvement of Gamma Interferon and Tumor Necrosis Factor in the Pathway of Suppression

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Experimental infections of mice with the African trypanosome *Trypanosoma brucei* lead to a profound state of T-cell unresponsiveness in the lymph node cell (LNC) compartment. This suppression is mediated by macrophage-like cells which inhibit interleukin 2 (IL-2) secretion and down-regulate IL-2 receptor expression (M. Sileghem, A. Darji, R. Hamers, M. Van de Winkel, and P. De Baetselier, Eur. J. Immunol. 19:829–835, 1989). Similar suppressive cells can be generated in vitro by pulsing 2C11-12 macrophage hybridoma cells with opsonized *T. brucei* parasites (2C11-12P cells). Cocultures of 2C11-12P cells and LNCs secrete higher levels of gamma interferon (IFN-γ), and the hyperproduction of IFN-γ was found to be confined to $CD8^+$ lymphoid cells. Elimination of $CD8^+$ cells from cocultures of 2C11-12P cells and LNCs restores the T-cell proliferative response. Furthermore, addition of neutralizing anti-IFN-γ antibodies to the cocultures reduces the level of suppression and concomitantly restores the level of IL-2 receptor expression. Hence, IFN-γ plays a cardinal role in this in vitro model for *T. brucei*-elicited immunosuppression. Cocultures of LNCs and 2C11-12P cells in a two-chamber culture system further demonstrated that cell-cell contact is required for hyperproduction of IFN-γ and, moreover, that IFN-γ cooperates with a 2C11-12P-derived diffusible factor to exert its suppressive activity. Finally, tumor necrosis factor alpha (TNF-α) produced by 2C11-12P cells was found to be implicated in the hyperproduction of IFN-γ, since addition of neutralizing anti-TNF-α antibodies to cocultures reduced the level of suppression and concomitantly abrogated the hyperproduction of IFN-γ. Collectively, our findings indicate that *T. brucei*-elicited suppressive 2C11-12 macrophage cells differentially influence T-cell subpopulations: (i) $CD8^+$ cells are signaled via cell-cell contact to produce IFN-γ, and TNF-α is implicated in this process, and (ii) locally produced IFN-γ and macrophage-released factors act in concert to inhibit $CD4^+$ and $CD8^+$ T-cell proliferative responses.

African trypanosomes are notorious as potent modulators of the host's immune system. Suppression of immune responses to parasite-unrelated antigens and mitogens during experimental trypanosome infections has frequently been described. However, regarding nonspecific immunosuppression of T-cell responses, there is no consensus about the nature of the mechanisms underlying the trypanosome-elicited immunosuppression. The reduced responsiveness has been attributed to suppressor T cells (18), suppressive macrophage-like cells (1), or combinations of the two (5).

Trypanosome-elicited suppressive macrophages seem to be crucial effector cells in the inhibition of the T-cell proliferative response in both experimental murine infections (1, 29) and natural bovine hosts (10). However, the mechanisms through which suppressive macrophages mediate their suppressive activity appear to be both tissue and infection stage dependent. Indeed, in the murine spleen, macrophages show an up-regulation of nitric oxide (NO) synthase activity, and locally released NO was found to exert an antiproliferative activity on lymphocytes (27, 32). The potential role for NO in the suppression of lymphocyte responses was recently substantiated in vivo by transfer experiments (20). While NO-dependent suppressive mechanisms were found to predominate in spleen and lymph node tissues during the early stage of *Trypanosoma brucei* infection (27, 32), we and others provided evidence that NO-independent mechanisms take over during the late stage of infection (6, 27). As far as lymph nodes are concerned, late-stage suppression appears to occur in an NO-independent but gamma interferon (IFN-γ)-dependent manner.

The molecular basis of lymph node T-cell unresponsiveness during late-stage *T. brucei* infections was analyzed in depth. Concanavalin A (ConA)-stimulated lymph node cells (LNC) from late-stage *T. brucei*-infected mice (LNCi) were found to be unable to secrete interleukin-2 (IL-2) and to express receptors for IL-2 (IL-2R) (31). Thus, in this experimental model, the inhibition of T-cell proliferation could be attributed to a suppression of the IL-2–IL-2R circuit. We and others have further shown that at least two unlinked mechanisms are implicated in the inhibition of IL-2 secretion and down-regulation of IL-2R (30). The generation of prostaglandin (PG)-secreting macrophages was found to be entirely responsible for the profound suppression of IL-2 production (29). In contrast, inhibition of IL-2R expression on both $CD4^+$ and $CD8^+$ target cells occurred in the presence of a potent PG inhibitor (i.e., indomethacin), demonstrating the involvement of a PG-inde-

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pendent mechanism in this mode of suppression (30). This second mechanism was, again, mediated by macrophage-like cells (29). The profound suppression of IL-2 production in the lymph node compartment does not reflect a complete paralysis of lymphokine secretion. Indeed, we have reported that LNCi were stimulated to produce high levels of IFN-γ (6, 31). Similarly, other investigators have demonstrated that *T. brucei* infections in rats induce IFN-γ secretion by $CD8^+$ T cells (3, 24, 25). Hence, *T. brucei* infections exert dual activities on the lymph node T-cell response: profound inhibition of T-cell proliferation and IL-2 secretion and induction of IFN-γ production. A link between these dual activities was established by demonstrating that IFN-γ is actively involved in the inhibition of IL-2R expression (6). Collectively, we have accumulated evidence that *T. brucei* infections elicit a novel suppressive mechanism that is NO independent but IFN-γ dependent in the lymph node compartment.

Recently, we have developed an in vitro model that allows the suppressive activity of *T. brucei*-elicited suppressive macrophage cells to be mimicked (28). This in vitro model consists of a macrophage hybridoma cell line (i.e., 2C11-12 cells), which upon interaction with opsonized living parasites exerts a suppressive activity on ConA-activated LNC. Interestingly, *T. brucei*-pulsed 2C11-12 cells (2C11-12P cells) failed to block IL-2 secretion in the presence of indomethacin but still suppressed IL-2R expression (28). Hence, 2C11-12P cells mediate a PG-independent suppressive mechanism similar to that occurring in the lymph nodes during infections with *T. brucei*.

We have further used the 2C11-12 model to unravel the IFN-γ-dependent mechanism of IL-2R suppression. The results demonstrate that via cell-cell contact, 2C11-12P cells stimulate $CD8^+$ T cells to produce IFN-γ and that tumor necrosis factor alpha (TNF-α) is implicated in this process. Furthermore, the released IFN-γ cooperates with a 2C11-12P-derived soluble component to inhibit T-cell proliferation.

MATERIALS AND METHODS

Animals and parasites. (C57BL/6 × BALB/c)F_1 mice (2 months old) purchased from the Studiecentrum voor Kernenergie (Mol, Belgium) were used in all the experiments. The pleomorphic *T. brucei* AnTat 1.1.E post fly clone, kindly provided by N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium), was used in all the experiments. Animals were inoculated intraperitoneally with 2×10^3 bloodstream-form parasites and survived approximately 35 days following infections. Pure parasite populations were prepared by chromatography on preswollen DEAE-Sepharose (Pharmacia, Uppsala, Sweden) as described elsewhere (14) and were stored in liquid nitrogen in a phosphate-saline-glucose buffer supplemented with 10% glycerol.

Reagents. N^G-monomethyl-L-arginine (L-NMMA) acetate salt was purchased from Calbiochem, San Diego, Calif., and used at 0.25 mM.

Nonpurified recombinant murine IFN-γ derived from the supernatant fluid of Mick cells, a CHO cell line expressing an amplified murine IFN-γ cDNA (9), was used as a source of exogenous IFN-γ in all experiments ($10^{4.5}$ IU/ml).

For in vitro neutralization of IFN-γ or TNF-α, neutralizing monoclonal rat antibody F3 (16) or 1F3F3 (19), respectively, was used.

Polyclonal rabbit antibodies elicited against the tip region of murine TNF-α (amino acids 99 to 115) were purified on protein G by a standard protocol and used to detect membrane-bound TNF-α (7).

2C11-12P cells. 2C11-12P cells were prepared according to a previously described procedure (28). Briefly, 2C11-12 cells were allowed to adhere to a plastic surface and subsequently incubated with antibody-coated, long slender *T. brucei* trypomastigotes at a macrophage-to-parasite ratio of 1:100 for 30 min. The cultures were then repeatedly washed until no free parasites could be observed by microscopy. 2C11-12P cells were released by vigorous pipetting, irradiated at 900 rads to stop their proliferation, and then cocultured with freshly isolated LNC at a final ratio of 1:10. This concentration corresponds roughly to the amount of macrophages found in LNC populations.

Proliferation assay. LNC and peritoneal exudate cells (PEC) from normal mice and from late-stage-infected mice (4 weeks postinfection [LNCi and PECi]) were used. Mesenteric and peripheral lymph nodes were removed aseptically, and single-cell suspensions were prepared in cold RPMI 1640 medium (Gibco, Grand Island, N.Y.). PEC were harvested by flushing the peritoneum with 10 ml of cold sucrose (0.34 M). After being washed, cells were cultured in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin-streptomycin (100 U and 100 μg/ml, respectively), L-glutamine (2 mM), and 2-mercaptoethanol (5×10^{-5} M).

For proliferation assays, aliquots containing 4×10^5 LNC were dispensed into flat-bottomed 96-well plates (Falcon) and cocultured in a final volume of 200 μl with 10% irradiated 2C11-12P cells in the presence of indomethacin (10 μg/ml) (Sigma, St. Louis, Mo.) to eliminate any suppressive activity due to PG. Cocultures were stimulated with ConA (2.5 μg/ml) (Sigma) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. About 18 h before harvesting, the cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) (TRA310; Amersham, Ghent, Belgium), and the mitogen-induced proliferative responses were determined by [^{3}H]TdR incorporation. Results were expressed as the mean [^{3}H]TdR incorporation from triplicate cultures with background values from nonstimulated cells subtracted. The background level of unstimulated cells was never higher than 5,000 cpm. All results are representative of independent experiments repeated at least three times.

In order to assess whether 2C11-12P cells affect antigen-specific T-cell proliferation, mice were injected via the footpads with 25 μg of keyhole limpet hemocyanin (KLH) (Calbiochem) emulsified in complete Freund adjuvant. Eight to 10 days later, popliteal LNC were prepared, cocultured with 2C11-12P cells, and restimulated with KLH (2.5 μg/ml). Antigen-driven proliferation was determined by [^{3}H]TdR incorporation.

Alternatively, cultures of 4×10^5 LNC or LNCi, cocultures of 2×10^5 LNC and LNCi, or cocultures of 4×10^5 LNC and 2×10^4 PEC or PECi were performed. ConA-induced proliferation was estimated as described above.

To estimate IFN-γ and NO production, cultures were grown in 24-well plates (Falcon) in a final volume of 1 ml, at concentrations equivalent to those used in 96-well plates. Cell supernatants were collected after 48 h and frozen at −80°C until use.

Cell depletions and isolations. Depletion and isolation of $CD4^+$ and $CD8^+$ lymphoid cells from LNC were performed by magnetic cell sorting using a magnetic separator (MACS), following a standardized separation protocol (MACS; Milteny Biotech GmbH). Briefly, 2×10^8 LNC resuspended in phosphate-buffered saline (PBS) containing 2% fetal calf serum were incubated for 10 min on ice with biotinylated anti-CD4 monoclonal antibody (GK 1.5) (American Type Culture Collection [ATCC], Rockville, Md.) or anti-CD8 monoclonal antibody (H35.17.2) (ATCC). After washings, the cells were incubated with fluorescein isothiocyanate-labeled streptavidin (Amersham) for 10 min on ice. The cells were washed with PBS containing 5 mM EDTA, resuspended, and incubated with biotinylated microbeads (MACS H 042103) for 5 min on ice. Finally, the cells were loaded on a cooled column (MACS, type-C), and the magnetic separation was carried out according to the instructions of the manufacturers. Adsorbed and nonadsorbed cell fractions were analyzed directly by flow cytometry for the expression of CD4 and CD8. Such populations were found to be either completely depleted or strongly enriched (90 to 95% pure) for the relevant markers.

IFN-γ determination. IFN-γ concentrations in cell-free supernatants were estimated in a sandwich enzyme-linked immunosorbent assay (ELISA). All reagents, including an international murine IFN-γ standard, were kindly provided by Innogenetics (Ghent, Belgium). Briefly, samples were incubated with immobilized monoclonal antibody specific for mouse IFN-γ (DB1 [34]) in microtiter plates (Maxisorb; Nunc). The bound cytokine was detected by consecutive incubation with biotinylated rabbit anti-mouse IFN-γ polyclonal antibodies and streptavidin-alkaline phosphatase conjugate (Amersham).

Quantification of nitrite. Nitrite was quantified by a standard Greiss reaction (15). Briefly, 100 μl of cell-free supernatants was added to an equal volume of Greiss reagent (0.5% sulfanilamide and 0.05% N-1 naphthylethylenediamine hydrochloride in 2.5% H_3PO_4). After 10 min of incubation at room temperature, the A_{540} was recorded. A standard curve was generated with known concentrations of $NaNO_2$ in culture medium. All reagents for nitrite assay were obtained from Sigma.

Expression of IL-2R. The number of IL-2R-positive cells (ConA-activated LNC) was assessed by standard fluorescence analysis using the monoclonal rat anti-mouse IL-2R antibody produced by 7D4 hybridoma (ATCC). Briefly, 100-μl aliquots of a lymphocyte suspension (10^7 cells per ml) were incubated (at 4°C for 30 min) with 10 μl of a 10-fold-concentrated culture supernatant of the 7D4 hybridoma, washed, and then incubated with 10 μl of a fluorescein isothiocyanate-labeled goat anti-rat antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.). The fluorescence activity was measured with a fluorescence-activated cell sorter (FACStar; Becton-Dickinson Electronic Lab Div., Mountain View, Calif.).

RESULTS

2C11-12P cells sensitize $CD8^+$ T cells for increased IFN-γ production. 2C11-12 cells, upon interaction with opsonized *T. brucei* parasites, acquire the capacity to inhibit ConA-induced T-cell blastogenic responses via a PG-independent mechanism (28). As shown in Fig. 1, the suppressive activity of 2C11-12P

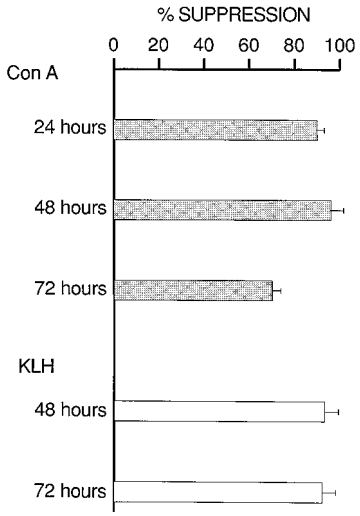

FIG. 1. Inhibition of ConA- or antigen-driven T-cell proliferation by 2C11-12P cells. Cocultures of 2C11-12P and LNC or KLH-specific LNC were stimulated with ConA or KLH, respectively, and pulsed with [^{3}H]TdR after 24, 48, or 72 h of culture. The proliferative responses of cocultures containing 2C11-12P cells were compared with those of cocultures containing 2C11-12 cells, and percent suppression was calculated. The amounts of [^{3}H]TdR incorporated in cocultures of ConA-activated LNC and 2C11-12 cells (expressed as change in counts per minute [Δcpm], in thousands) were 168 ± 12, 174 ± 13, and 159 ± 10, respectively, after 24, 48, and 72 h, and those incorporated in cocultures of KLH-restimulated specific LNC and 2C11-12 cells were 56 ± 4 and 62 ± 3, respectively, after 48 and 72 h.

cells on ConA-activated LNC is maintained throughout the cycle of the proliferative response, indicating that the reduced proliferation does not reflect an alteration of the response kinetics. Furthermore, the suppressive activity of 2C11-12P cells is not restricted to ConA-activated cells, since similar levels of suppression were recorded for antigen-specific (i.e., KLH-driven) LNC proliferation (Fig. 1).

Since IFN-γ was reported to be involved in *T. brucei*-elicited immunosuppression (6), it was of interest to quantify IFN-γ in the supernatants of ConA-activated cocultures of LNC and 2C11-12P cells. As shown in Fig. 2, ConA-stimulated LNC produced significantly higher levels of IFN-γ when cultured with 2C11-12P cells than when cultured by themselves or with 2C11-12 cells. Sensitization for IFN-γ synthesis was already recorded after 12 h of cocultures and reached maximal levels after 24 h (not shown). Hence, under these experimental conditions, a decreased T-cell proliferation is paralleled by an increased IFN-γ production. To determine the phenotype of the cells responsible for IFN-γ production, LNC populations were separated in specific T-cell populations (either $CD8^+$ or $CD4^+$) and the depleted or isolated subpopulations were analyzed for IFN-γ production. Following coculture with 2C11-12P cells and stimulation with ConA over a period of 12 h, depletion of $CD8^+$ cells but not $CD4^+$ cells resulted in a complete abolishment of the hyperproduction of IFN-γ in LNC populations (Fig. 2). Conversely, ConA-stimulated $CD8^+$ cells but not $CD4^+$ cells produced increased amounts of IFN-γ when cultured with 2C11-12P cells. Collectively, these experiments demonstrate that 2C11-12 macrophage-like cells acquire, upon interaction with *T. brucei*, the potential to trigger ConA-sensitized $CD8^+$ LNC to produce IFN-γ.

Involvement of IFN-γ in the suppressive activity of 2C11-12P cells. The observed inverse effects of 2C11-12P cells on T-cell proliferation (inhibition) and IFN-γ secretion (stimulation) raised the question of whether a local hyperproduction of

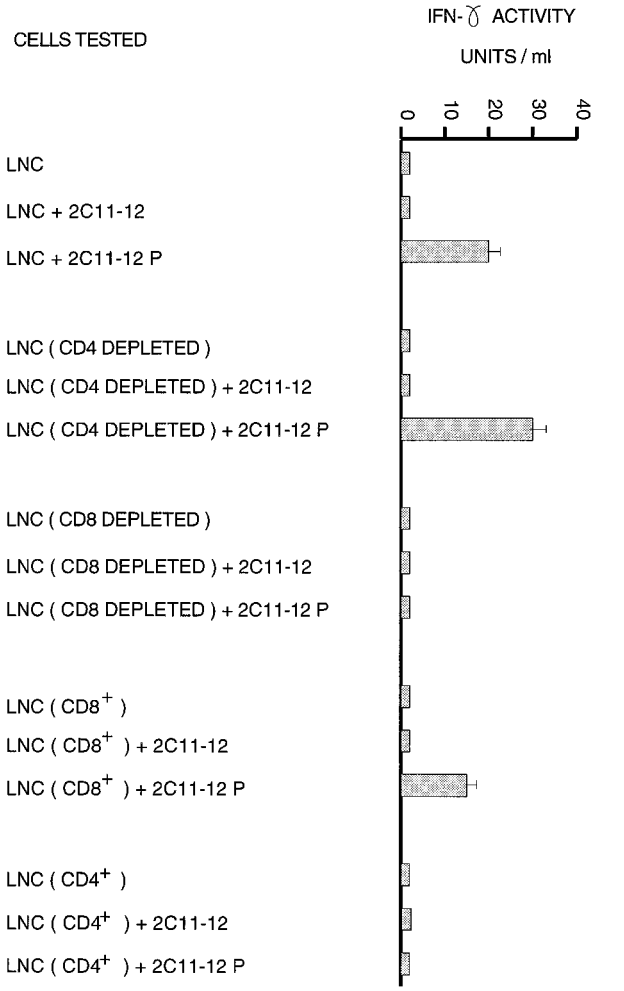

FIG. 2. IFN-γ production by $CD4^+$ or $CD8^+$ subpopulations of LNC. LNC, depleted, and purified ($CD4^+$ or $CD8^+$) populations were stimulated with ConA, and supernatants were tested for IFN-γ activity by ELISA. Data are the means of triplicate experiments. Standard deviations (error bars) are shown when exceeding 10% of the mean.

IFN-γ contributes to the suppression of T-cell proliferation. Since $CD8^+$ cells were identified as the major source of IFN-γ production in cocultures of LNC and 2C11-12P cells, the suppressive activity of 2C11-12P cells on LNC depleted for either $CD8^+$ or $CD4^+$ cells was tested (Table 1). The results clearly demonstrate that $CD8^-$ $CD4^+$ LNC populations are not sus-

TABLE 1. 2C11-12P cells exert a suppressive activity via $CD8^+$ cells

Cells[a]	Proliferation (Δcpm, 10^3) in the presence of:		
	Medium alone	2C11-12 cells	2C11-12P cells
LNC	96 ± 7	128 ± 9	36 ± 10[b]
LNC ($CD8^-$ $CD4^+$)	61 ± 5	73 ± 12	58 ± 2
LNC ($CD8^+$ $CD4^-$)	48 ± 4	56 ± 7	11 ± 2[b]

[a] Total LNC populations or LNC depleted for either $CD8^+$ ($CD8^-$ $CD4^+$) or $CD4^+$ ($CD8^+$ $CD4^-$) T cells were cocultured with 2C11-12 or 2C11-12P cells and stimulated with ConA to evaluate the suppressive activity of such cells on different LNC populations.

[b] Proliferative response significantly lower than the LNC response (two-tailed Student's *t* test; $P < 0.001$).

TABLE 2. Involvement of IFN-γ in the suppressive activity of 2C11-12P cells

Expt and culture[a]	Proliferation (Δcpm, 10^3) in the presence of:	
	Medium alone	Anti-IFN-γ[b]
1		
LNC	155 ± 13	171 ± 25
LNC + 2C11-12P cells	50 ± 6[c]	139 ± 23
2		
LNC	161 ± 9	200 ± 21
LNC + 2C11-12P cells	78 ± 12[c]	201 ± 20

[a] ConA-stimulated cocultures of LNC and 2C11-12P cells were performed to evaluate the suppressive activity of such cells on the response of LNC.
[b] Anti-IFN-γ antibodies (10^3 neutralizing units per ml) in the cultures.
[c] Proliferative response significantly lower than LNC response (two-tailed Student's *t* test; $P < 0.001$).

ceptible to the antiproliferative activity of 2C11-12P cells. In contrast, $CD8^+$ $CD4^-$ LNC populations show an impaired proliferative response when cocultured with 2C11-12P cells. These results suggest that IFN-γ-producing $CD8^+$ cells play a major role in the suppression of T-cell proliferation. To further assess the possible involvement of IFN-γ in the impaired proliferative response in cocultures of LNC with 2C11-12P, neutralizing anti-IFN-γ antibodies were used. The results compiled in Table 2 indicate that the presence of anti-IFN-γ during ConA activation almost completely restored the proliferative response in cocultures of LNC and 2C11-12P. These experiments demonstrate that locally produced IFN-γ participates in the suppression of T-cell proliferation. The influence of anti-IFN-γ antibodies on the expression of IL-2R on ConA-activated LNC cocultured with 2C11-12P cells was studied by fluorescence-activated cell sorter analysis. According to the fluorescence profiles shown in Fig. 3, 2C11-12P cells induce a shift in the density of IL-2R expression on LNC towards weakly fluorescent cells (Fig. 3A). The presence of anti-IFN-γ antibodies in these cocultures resulted in a significant reexpression of IL-2R (Fig. 3B), demonstrating that locally produced IFN-γ contributes to a down-regulation of IL-2R on LNC and consequently to impaired T-cell proliferative responsiveness.

2C11-12P cells mediate suppression via cell-cell contact and soluble components. Thus far, suggestive evidence was provided that IFN-γ, produced during interactions between 2C11-12P cells and LNC, participates in the suppression of ConA-induced T-cell proliferation. The role of IFN-γ in the pathway of suppression was further investigated, and different possibilities were considered.

IFN-γ may interfere directly with the activation of LNC. This possibility is unlikely, since addition of exogenous recombinant IFN-γ (up to 1,000 U/ml) to ConA-activated LNC did not result in an inhibition of the proliferative response (not shown).

IFN-γ produced in coculture experiments might activate 2C11-12P cells towards a suppressive potential, such as induction of NO release. However, ConA-activated cocultures of LNC and 2C11-12P did not produce substantial amounts of NO, in contrast to cocultures of ConA-activated LNC and PECi (Table 3). Furthermore, addition of the arginine NO synthase inhibitor L-NMMA did not abrogate the suppressive activity of 2C11-12P cells while inhibiting completely the suppressive activity of PECi (not shown). Thus, these experiments failed to provide evidence for a suppressive role of IFN-γ via up-regulation of NO release and for the involvement of NO in the suppressive activity of 2C11-12P cells.

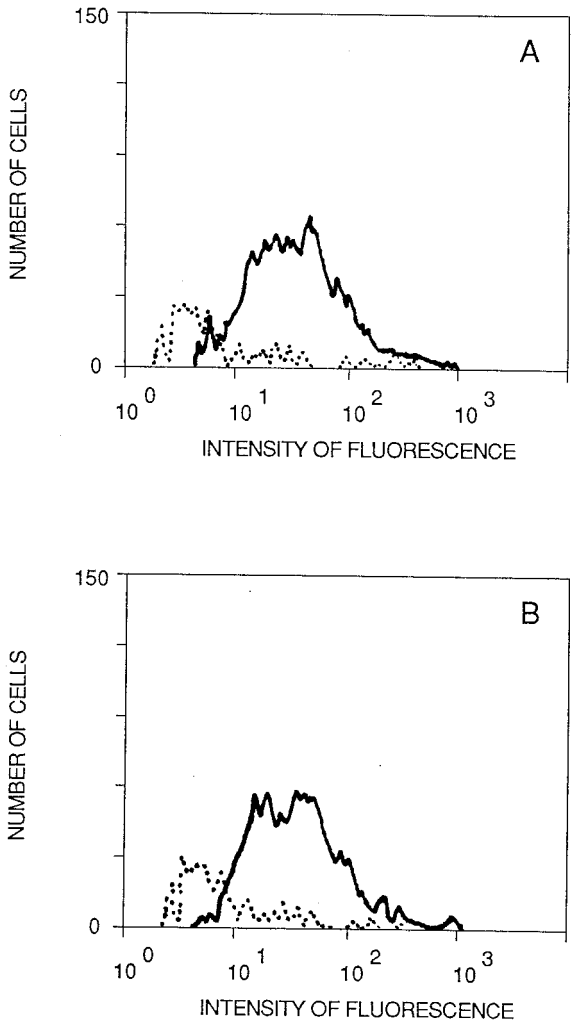

FIG. 3. Restoration of IL-2R expression by anti-IFN-γ antibodies. (A) IL-2R expression on ConA-stimulated LNC (solid line) or LNC cocultured with 2C11-12P (broken line). (B) IL-2R expression on ConA-stimulated cocultures of LNC and 2C11-12P cells in the absence (broken line) or presence (solid line) of anti-IFN-γ antibodies. The profile in the absence of anti-IFN-γ antibodies is identical to the one shown in panel A.

IFN-γ might require the presence of additional factors secreted by 2C11-12P cells to exert a suppressive activity. To test this possibility, coculture experiments were performed in Marbrook chambers in which LNC and 2C11-12P or 2C11-12 cells were placed in separate chambers (Table 4). Under these experimental conditions, there was no suppression of ConA-induced proliferation, indicating that cell-cell contact between 2C11-12P cells and LNC is a prerequisite for induction of suppression. Furthermore, under such coculture conditions, there was no increased IFN-γ production (not shown). However, addition of exogenous IFN-γ to the Marbrook cultures resulted in a significant level of suppression in cocultures of LNC with 2C11-12P cells but not in those with 2C11-12 cells (Table 4). These results suggest that at least two diffusible mediators are simultaneously required to mediate an inhibitory activity, namely, IFN-γ and a factor(s) released by 2C11-12P cells.

2C11-12P cells stimulate increased IFN-γ production via TNF-α. It has been reported previously that *T. brucei* components activate 2C11-12 cells to produce TNF-α (21). Further-

TABLE 3. Suppressive activity of 2C11-12P cells is not mediated by nitric oxide

Culture	L-NMMA[a]	Proliferation (Δcpm, 10^3)	NO production (μM)
LNC	−	87 ± 7	3.2 ± 0.9
	+	92 ± 6	2.0 ± 0.2
LNC + 2C11-12 cells	−	100 ± 9	1.7 ± 1.4
	+	102 ± 7	1.3 ± 0.1
LNC + 2C11-12P cells	−	55 ± 3[b]	4.7 ± 0.8
	+	54 ± 5[b]	1.9 ± 0.4
LNC	−	286 ± 18	3.2 ± 1.0
	+	287 ± 13	1.0 ± 0.2
LNC + PEC	−	278 ± 5	3.5 ± 0.9
	+	290 ± 12	0
LNC + PECi	−	5 ± 1[b]	20.3 ± 1.7[c]
	+	274 ± 13	2.4 ± 0.3

[a] Presence of 0.25 mM L-NMMA in the cultures.
[b] Proliferative response significantly lower than LNC response (two-tailed Student's *t* test; $P < 0.001$).
[c] NO production significantly higher than LNC response (two-tailed Student's *t* test; $P < 0.01$).

more, by using anti-TNF-α antibody that allows the detection of membrane-bound TNF-α (7), it could be demonstrated that the expression of membrane-bound TNF-α is up-regulated on 2C11-12 cells upon interaction with trypanosomes (mean fluorescence intensities of 366 for 2C11-12P cells versus 150 for 2C11-12 cells). It was thus of interest to test whether TNF-α contributes to the suppressive activity of 2C11-12P cells. The presence of neutralizing monoclonal anti-TNF-α antibodies during ConA activation of cocultures of LNC and 2C11-12P significantly restored the proliferative responses of the cocultures (Table 5). These results indicate that TNF-α participates in the suppression of T-cell proliferation. This observation is relevant for the in vivo situation, since neutralizing anti-TNF-α antibodies also restored the proliferative response of cocultures of LNC and LNCi (Table 5).

The involvement of TNF-α in the pathway of suppression is not due to a direct or indirect suppressive activity on LNC, since addition of exogenous recombinant TNF-α (up to 1,000 U/ml), either alone or combined with IFN-γ, to ConA-activated LNC did not result in an inhibition of the proliferative response (not shown). The possibility that TNF-α could play a role in the sensitization of LNC for increased IFN-γ production was then considered. As shown in Fig. 4, neutralizing anti-TNF-α antibodies inhibited completely the capacity of 2C11-12P cells to sensitize ConA-activated LNC to produce IFN-γ, while no effect was observed with an isotype-matched control. Hence, TNF-α contributes to the suppressive activity of 2C11-12P cells by inducing local IFN-γ production in the cocultures.

TABLE 4. Suppressive activity of 2C11-12P cells: contributions of cell contact and soluble factors

Expt and culture[a]	Proliferation (Δcpm, 10^3) in the presence of:	
	Medium alone	IFN-γ[b]
1		
LNC	135 ± 11	150 ± 26
LNC + 2C11-12 cells	133 ± 15	120 ± 7
LNC + 2C11-12P cells	140 ± 24	60 ± 7[c]
2		
LNC	58 ± 9	68 ± 14
LNC + 2C11-12 cells	56 ± 14	52 ± 6
LNC + 2C11-12P cells	64 ± 12	16 ± 3[c]

[a] LNC were cultured in the bottom compartment of a Marbrook chamber and stimulated with ConA. 2C11-12P or 2C11-12 cells were added in the upper compartment of the Marbrook chamber to evaluate the suppressive activity of soluble factors.
[b] Presence of IFN-γ (10^2 U/ml) in the cultures.
[c] Proliferative response significantly lower than the LNC response (two-tailed Student's *t* test; $P < 0.001$).

TABLE 5. TNF-α contributes to the suppressive activity of 2C11-12P cells

Culture[a]	Proliferation (Δcpm, 10^3) in the presence of:	
	Medium alone	Anti-TNF-α[b]
LNC	156 ± 15	187 ± 18
LNC + 2C11-12 cells	170 ± 12	210 ± 20
LNC + 2C11-12P cells	20 ± 2[c]	122 ± 9[c]
LNC	140 ± 7	105 ± 9
LNCi	58 ± 4[c]	70 ± 6[c]
LNC + LNCi	60 ± 5[c]	174 ± 12

[a] Cocultures of LNC and 2C11-12P cells or LNC and LNCi were stimulated with ConA.
[b] Presence of 10^3 neutralizing U of anti-TNF-α antibodies per ml.
[c] Proliferative response significantly lower than LNC response (two-tailed Student's *t* test; $P < 0.005$).

DISCUSSION

We and others have previously demonstrated that the suppression of T-cell proliferative responses during *T. brucei* infections is attributable to the presence of macrophage-like suppressive cells that block IL-2 production through a PG-dependent mechanism and block IL-2R expression through a PG-independent mechanism (29). We assumed that macrophages acquire this suppressive potential following in vivo interaction with *T. brucei* parasites. This assumption was based on the observation that 2C11-12 macrophage hybridoma cells pulsed with opsonized *T. brucei* exhibit a suppressive activity similar to that of in vivo-elicited macrophages (28).

The simultaneous suppression of IL-2 production and IL-2R expression does not reflect a total unresponsiveness of mitogen-stimulated T cells. Indeed, we have reported previously that ConA-activated LNCi populations were perfectly able to secrete lymphokines other than IL-2, such as IFN-γ (6, 31). Accordingly, our present results demonstrate that coculture of LNC with *T. brucei*-elicited suppressive cells (2C11-12P) does not result in impaired IFN-γ production. Rather, these suppressive cells induce increased IFN-γ production. Hence, under these experimental conditions, inhibition of T-cell proliferation is paralleled by increased lymphokine production (i.e., IFN-γ). It is possible that trypanosomes modify antigen-presenting cells (such as macrophages) in such a way that signalling of T cells results in lymphokine secretion rather than proliferation. Indeed, according to a recent report, trypanosome infections sensitize T-cell responses to the variant surface glycoprotein (VSG) (26). These VSG-specific T-cell responses exhibit evidence of clonal maturation (lymphokine production) but not clonal expansion (proliferation) after antigenic challenge. Interestingly, the pattern of lymphokine secretion of VSG-responsive T cells indicates that VSG stimulates preferentially a TH1 cell subset during infection. In our experimental

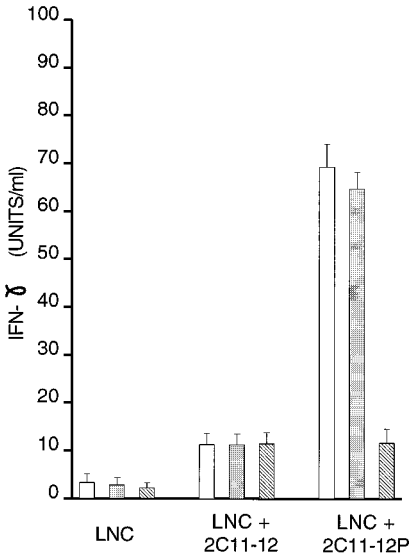

FIG. 4. Anti-TNF-α antibodies inhibit the ability of 2C11-12P cells to stimulate ConA-activated LNC to produce IFN-γ. ConA-stimulated cocultures of LNC and 2C11-12 or 2C11-12P cells were performed in the presence of anti-TNF-α antibodies, and IFN-γ production was determined in cell-free supernatants. □, no antibodies; ▧, anti-TNF-α antibodies; ▨, isotype-matched control.

system, the hyperproduction of IFN-γ in LNC was found to be confined to $CD8^+$ T cells, suggesting that this subpopulation is responsive to a putative costimulatory signal of 2C11-12P cells. This costimulatory activity may be mediated by TNF-α, since anti-TNF-α antibodies abrogated completely the increased IFN-γ production in cocultures of 2C11-12P cells and ConA-activated LNC. Since increased IFN-γ production required physical contact between 2C11-12P cells and responding $CD8^+$ T cells, membrane-bound TNF-α expressed on 2C11-12P cells could function as a costimulator. In fact, membrane-bound monokines such as TNF-α and IL-1 were reported to provide costimulatory activity during T-cell activation (35). However, although 2C11-12P cells express higher levels of membrane-bound TNF-α than 2C11-12 cells, the amount of membrane-bound TNF-α expressed on 2C11-12 cells is not negligible, and yet these cells did not induce IFN-γ production. Hence, membrane-bound TNF-α exposed on 2C11-12 cells may be required yet not sufficient to sensitize $CD8^+$ T cells for IFN-γ synthesis. Although the enhanced IFN-γ production by $CD8^+$ cells required in vitro mitogenic stimulation, a similar hyperproduction might also occur in vivo following stimulation with parasite antigens. Indeed, rats infected with *T. brucei* show a strong and rapid increase in the number of IFN-γ-producing cells, and these IFN-γ-producing cells were identified as $CD8^+$ lymphoid cells (3, 25). Moreover, it was recently reported that *T. brucei*-released factors can trigger a rapid release of IFN-γ by $CD8^+$ cells in a non-antigen-specific way (2). Hence, there is clear evidence that infections with *T. brucei* sensitize the immune system for IFN-γ production both in vivo and in vitro.

A high level of local production of IFN-γ might in turn affect immune responsiveness. Indeed, IFN-γ has been reported to down-regulate immunity, including inhibition of delayed-type hypersensitivity (8) and antigen-specific proliferation (22). Furthermore, in addition to its potent antiproliferative activity on certain cell types (11, 13, 23), IFN-γ also exerts an inhibitory effect on antibody (4) and lymphokine (12) production. Collectively, these multiple activities of IFN-γ demonstrate its critical role in maintaining the homeostasis of the immune response. Accordingly, our previous and present results point to the involvement of IFN-γ in *T. brucei*-elicited suppression of T-cell proliferative responses. In an earlier report, evidence that IFN-γ participates in both the inhibition of IL-2R expression and the generation of suppressive cells during infections with *T. brucei* was provided (6). The role of IFN-γ in the manifestation of suppression is corroborated here in the 2C11-12 model, since addition of anti-IFN-γ antibodies to cocultures of suppressive cells and mitogen-stimulated LNC restored T-cell proliferation and IL-2R expression.

Though the experimental data suggest that IFN-γ plays a central role in the pathway of immunosuppression, this cytokine does not seem to be the sole effector of suppression. First, addition of exogenous IFN-γ to ConA-stimulated LNC did not result in an inhibition of T-cell proliferation. Second, the experiments with Marbrook chamber cultures demonstrate that IFN-γ is required yet not sufficient to inhibit mitogen-induced T-cell proliferation. In fact, the data indicate that IFN-γ cooperates with a 2C11-12P-derived soluble factor in the inhibition of T-cell proliferative responses. Similar observations were reported for graft-versus-host reaction-associated immunosuppression, in which it was shown that IFN-γ requires other molecules for the inhibitory activity of graft-versus-host reaction-suppressive cells (17).

Collectively, the data accumulated so far for *T. brucei*-elicited suppression in vitro and in vivo point towards the following pathway for trypanosome-elicited suppression of IL-2R expression: (i) trypanosomes or trypanosome fragments interact with macrophages; (ii) trypanosome-pulsed or -triggered macrophages provide, through cell-cell contact, a costimulatory signal (involving TNF-α) to $CD8^+$ T cells, which secrete substantial amounts of IFN-γ upon triggering by external stimuli (mitogens and antigens); (iii) the produced IFN-γ and soluble factors, released by trypanosome-pulsed or -triggered macrophages, act in concert to inhibit IL-2R expression on $CD4^+$ and $CD8^+$ cells.

Recent reports have documented a clear role for macrophage-derived NO in suppression of proliferative T-cell responses in trypanosomiasis (27, 32). Furthermore, the up-regulation of NO synthesis was found to be partially dependent upon IFN-γ and TNF-α. On the basis of these observations, it was proposed that trypanosome infections stimulate immune cells to produce IFN-γ and TNF-α, which in turn activate macrophages to produce the antiproliferative component NO (32). Though the 2C11-12 model confirms the role of IFN-γ and TNF-α in the pathway of suppression, these cytokines do not operate only via regulation of NO synthesis. Indeed, we could not provide substantive evidence for a role of NO in the effector phase of suppression. First, 2C11-12P cells do not produce significant levels of NO, either alone or in coculture with ConA-activated LNC. This does not reflect the inability of 2C11-12 cells to produce NO, since this cell line is perfectly able to secrete NO when stimulated with lipopolysaccharide and IFN-γ as well as with trypanosome components (not shown). However, in our experimental setup, 2C11-12 cells were irradiated and, as reported previously (33), upon irradiation macrophages become unable to secrete NO. Second, the suppressive activity of 2C11-12P cells could not be abrogated by L-NMMA, a competitive inhibitor of arginine-dependent NO synthase (not shown), and similarly, this agent did not inhibit the suppressive activity of lymph node suppressive cells (6). Since the suppressive role for NO in trypanosomiasis was documented mainly for spleen cells and PEC (27, 32), it may well be that trypanosome infections elicit different types of suppressive mechanisms in distinct lymphoid compartments. In fact, we have identified a NO-independent but IFN-γ-depen-

dent mechanism of suppression that operates only in the lymph nodes during the late stage of *T. brucei* infections (3a). The 2C11-12 model described here mimics perfectly the NO-independent mechanism underlying lymph node suppression and will be further adopted to unravel this pathway of suppression.

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