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Inhibition of T-Cell Responsiveness during Experimental Infections with *Trypanosoma brucei*: Active Involvement of Endogenous Gamma Interferon

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Lymph node cells (LNC) from mice infected with *Trypanosoma brucei* contain macrophage-like cells that inhibit interleukin-2 receptor (IL-2R) expression (M. Sileghem, A. Darji, R. Hamers, M. Van De Winkel, and P. De Baetselier, Eur. J. Immunol. 19:829–835, 1989). Evidence that gamma interferon (IFN-γ) is actively involved in (i) the inhibition of IL-2R expression and (ii) the generation of suppressive cells during infections with *T. brucei* is presented. First, despite an impaired T-cell mitogenic response, LNC from infected mice are hyperresponsive for IFN-γ production. Second, addition of neutralizing anti-IFN-γ antibodies to cocultures of normal LNC and suppressive LNC populations reduces the level of suppression and restores the level of IL-2R expression. Third, administration of anti-IFN-γ to *T. brucei*-infected animals increases the blastogenic response and reduces the suppressive activity of LNC.

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 (18). This suppression was found to be mediated by macrophage-like cells which inhibit interleukin-2 (IL-2) production via prostaglandin secretion and inhibit IL-2 receptor (IL-2R) expression via a prostaglandin-independent mechanism (17). The simultaneous suppression of IL-2 production and IL-2R expression does not reflect a total unresponsiveness of mitogen-activated T cells. Indeed, we have reported that LNC from *T. brucei*-infected mice (LNCi) were perfectly able to secrete lymphokines other than IL-2, such as gamma interferon (IFN-γ) (19). Other investigators have demonstrated that infections of rats with *T. brucei* induce a strong and rapid increase in the number of IFN-γ-producing cells of the $CD8^+$ phenotype (1). Thus, it appears that infections with *T. brucei* exert dual activities on the T-cell response: profound inhibition of T-cell proliferation and sensibilization for IFN-γ production. In view of the multiple immunosuppressive activities of IFN-γ (3, 11, 12), it was of interest to analyze whether IFN-γ is involved, as a mediator, in the *T. brucei*-elicited impairment of T-cell proliferation.

The pleomorphic *T. brucei* AnTat 1.1.E clone, kindly provided by N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium), was used in all the experiments. $(C57BL/6 \times BALB/c)F_1$ mice (4 to 5 months old) were infected with *T. brucei* by intraperitoneal administration of 2×10^5 parasites. Suppression of T-cell responsiveness in *T. brucei*-infected mice reaches its peak at week 3 after infection (19). Therefore, in all the experiments the LNC from infected mice were isolated at week 4 after infection.

LNC populations were prepared in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Boehringer Pharma, Mannheim, Germany), 50 U of penicillin-streptomycin per ml, 300 μg of L-glutamine per ml, and 5×10^{-5} M 2-mercaptoethanol. Indomethacin (Sigma, St. Louis, Mo.) (10 μg/ml) was added to all the cultures in order to eliminate any suppressive activity due to prostaglandins. For proliferation assays, aliquots containing 2×10^5 or 4×10^5 viable cells (2×10^6/ml) were dispensed into flat-bottom 96-well microtiter plates (Nunc, Roskilde, Denmark) with or without 2.5 μg of concanavalin A (ConA) (Sigma) per ml. The cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. About 18 h before harvest, the cultures were pulsed with 1 μCi of [*methyl*-^{3}H]thymidine purchased from Amersham (Buckingshamshire, United Kingdom) and the mitogen-induced proliferative responses were determined by [^{3}H]thymidine incorporation. Cultures were performed in triplicate, and the proliferative responses are reported as change in counts per minute (Δcpm, in thousands) where Δcpm = mean cpm for stimulated cells − mean cpm for unstimulated cells. For determinations of macrophage-activating factors (MAF) and IFN-γ, 1-ml aliquots containing 2×10^6 viable cells were dispensed in flat-bottom 24-well plates (Nunc) and were stimulated with 5 μg of ConA per ml. The cultures were then incubated at 37°C in a humidified atmosphere containing 5% CO_2.

The presence of MAF lymphokines in the cell-free supernatant was determined by their capacity to prime the chemiluminescent reactivity of a cloned macrophage hybridoma cell line (2C11-12) as described previously (2). MAF activity is represented as the stimulation index: the chemiluminescent response (peak chemiluminescence value) of activated 2C11-12 cells divided by the chemiluminescent response of nonactivated 2C11-12 cells. The IFN-γ activity in LNC cultures was evaluated by using a mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) based on two monoclonal antibodies (MAbs): the mouse anti-rat IFN-γ MAb DB1 (21) and the rat anti-mouse IFN-γ MAb F1 (9).

The number of $IL\text{-}2R^+$ cells (ConA-activated LNC) was assessed by standard fluorescence analysis using the rat anti-mouse IL-2R MAb produced by the 7D4 hybridoma

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(American Type Culture Collection, Rockville, Md.). Briefly, 100-μl aliquots of a lymphocyte suspension (10^7 cells per ml) were incubated with 10 μl of a 10-fold-concentrated culture; supernatants of the 7D4 hybridoma were washed and then incubated with 10 μl of a fluorescein isothiocyanate-labeled goat anti-rat immunoglobulin antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.) at 4°C for 30 min. The fluorescence activity was measured on a fluorescence-activated cell sorter (FACStar; Becton-Dickinson Electronic Lab, Mountain View, Calif.).

All experiments described here were repeated at least twice and found to be consistently reproducible.

For in vivo and in vitro neutralization of endogenous murine IFN-γ, the neutralizing rat MAb F3 was used. The potential of this antibody to bind IFN-γ and to neutralize antiviral effects has been described elsewhere (9). The antibody was obtained as ascitic fluid from thymusless nude mice (*nu/nu*). The neutralizing batch used in the present study was $10^{-5.4}$ against 30 U of murine IFN-γ per ml (rat immunoglobulin G content, 1.6 mg/ml). Groups of F_1 mice (three animals) were treated with ascitic fluid with the neutralizing anti-IFN-γ MAb F3 according to the following scheme of administration: (i) intraperitoneal inoculation of 100 μl of F3 ascitic fluid 6 h before inoculation with 2×10^5 *T. brucei* organisms and (ii) intraperitoneal inoculation of 100 μl of F3 ascitic fluid 6 days after the inoculation with 2×10^5 *T. brucei* organisms. Control groups consisted of anti-IFN-γ treated noninfected mice and anti-IFN-γ untreated noninfected mice.

Supernatants of ConA-activated LNC, LNCi, and cocultures of LNCi with LNC were tested for their capacity to prime the bioluminescent activity of a macrophage hybridoma cell line. This macrophage activation assay proved to be reliable for the detection and quantification of MAF such as IFN-γ (2). As shown in Fig. 1A, LNCi as well as cocultures of LNCi with LNC produced an increased and accelerated MAF response compared with that of LNC. In characterization experiments, this MAF activity was found to be completely blocked by neutralizing anti-IFN-γ antibodies. Furthermore, by means of an IFN-γ-specific ELISA, enhanced IFN-γ production was detected in the supernatant of LNCi and cocultures of LNC and LNCi (Fig. 1B). Testing the proliferative capacity of the different cell populations revealed that LNCi and cocultures of LNCi and LNC were significantly reduced in ConA-induced T-cell blastogenic responsiveness compared with LNC (LNC, 186×10^3 cpm; LNCi, 4×10^3 cpm; LNC plus LNCi, 18×10^3 cpm). Thus, experimental *T. brucei* infections cause impairment of the T-cell proliferative response and concomitantly hyperresponsiveness for MAF–IFN-γ production in LNC populations.

To assess the potential involvement of IFN-γ in the impaired proliferative response of cocultures of LNCi with LNC, neutralizing antibodies were added to the cocultures. The results (Table 1) demonstrate that anti-IFN-γ antibodies almost completely restored the proliferative response of the cocultures. The influence of anti-IFN-γ antibodies on the expression of IL-2R on ConA-activated LNC and cocultures of LNC with LNCi was studied by fluorescence-activated cell sorter analysis. According to the fluorescence profiles shown in Fig. 2, LNCi induce a shift in the density of IL-2R expression on LNC towards cells with low-level fluorescence. The presence of anti-IFN-γ antibodies in these cocultures allowed a significant up-regulation of IL-2R. Collectively, these results demonstrate that locally produced

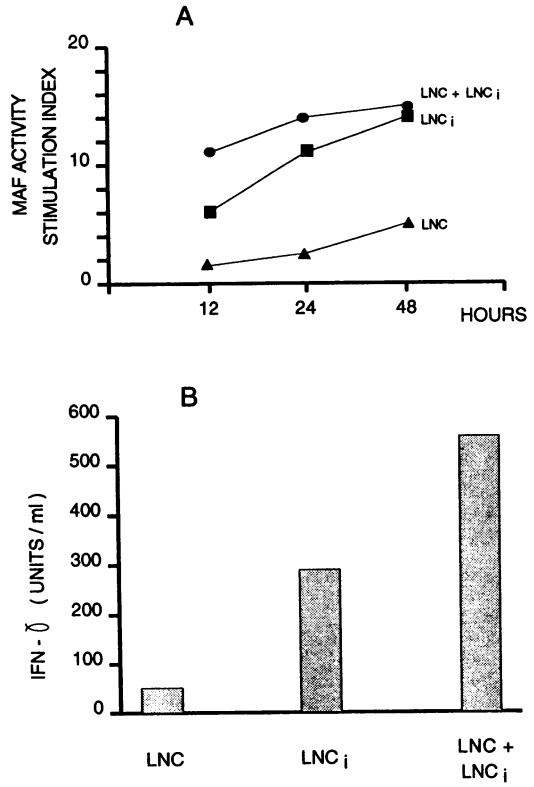

FIG. 1. (A) Kinetics of MAF production by ConA-stimulated LNC, LNCi, and cocultures of LNC with LNCi. MAF activity in the supernatants of the indicated cell populations was determined at different time intervals by a chemiluminescent bioassay (see text). (B) IFN-γ production by ConA-stimulated LNC, LNCi, and cocultures of LNC with LNCi. IFN-γ activity at 24 h in the supernatants of the indicated cell populations was determined by an IFN-γ specific ELISA.

IFN-γ contributes to a down-regulation of IL-2R on LNC and consequently to a reduced T-cell proliferative response.

To further substantiate the role of IFN-γ in the induction of immunosuppression during infections with *T. brucei*, anti-IFN-γ antibodies were administered to *T. brucei*-infected mice (either 6 h before or 6 days after infection) and LNCi were tested for their proliferative response to ConA on day 30 after infection. The results, shown in Table 2, demonstrate that treatment with anti-IFN-γ during infections with *T. brucei* substantially restores the ConA-induced proliferative responsiveness of LNCi. In contrast, the impaired proliferative response of spleen cells from *T. brucei*-infected animals (SPCi) is unaffected by anti-IFN-γ treatment (Table 2). These results indicate that LNC but not spleen cells (SPC) require IFN-γ in vivo for induction of unresponsiveness. Interestingly, LNCi derived from anti-IFN-γ treated mice failed to exert a suppressive activity on LNC (Table 3), indicating a crucial role for IFN-γ in the generation of suppressive cells during infections with *T. brucei*. In order to evaluate whether the anti-IFN-γ treatment affects, to some extent, the development of *T. brucei* parasites, parasitemia was monitored in the infected animals that were either untreated or treated with anti-IFN-γ. Anti-IFN-γ treatment was found to reduce by 50% parasite development during the first peak of parasitemia. Subsequent waves of parasitemia were, however, similar in con-

TABLE 1. Involvement of IFN-γ in the suppressive activity of LNCi

Expt and culture[a]	Anti-IFN-γ treatment[b]	Proliferation (Δcpm, 10^3)	% Reduction[c]
1			
LNC	−	153 ± 15	
LNC + LNCi	−	20 ± 3[d]	87
LNC	+	120 ± 6	
LNC + LNCi	+	116 ± 17	4
2			
LNC	−	185 ± 14.7	
LNC + LNCi	−	12 ± 4[d]	94
LNC	+	163 ± 9	
LNC + LNCi	+	188 ± 9	0

[a] LNC cultured at a concentration of 2 × 10^6/ml were stimulated with ConA. Cocultures of LNC (2 × 10^6/ml) and LNCi (2 × 10^6/ml) were made to evaluate the suppressive activity of LNCi on the response of LNC. In all experiments, a control containing twice the amount of LNC (4 × 10^6/ml) was included to evaluate the influence of cell crowding on T-cell responsiveness, and a negative influence of cell crowding on the proliferative response was never observed.

[b] Presence (+) or absence (−) of anti-IFN-γ antibodies (10^3 neutralizing units per ml) in the cultures.

[c] Percent reduction was calculated relative to the proliferation of LNC.

[d] Proliferative response significantly lower than the LNC response (two-tailed Student's test, $P < 0.001$).

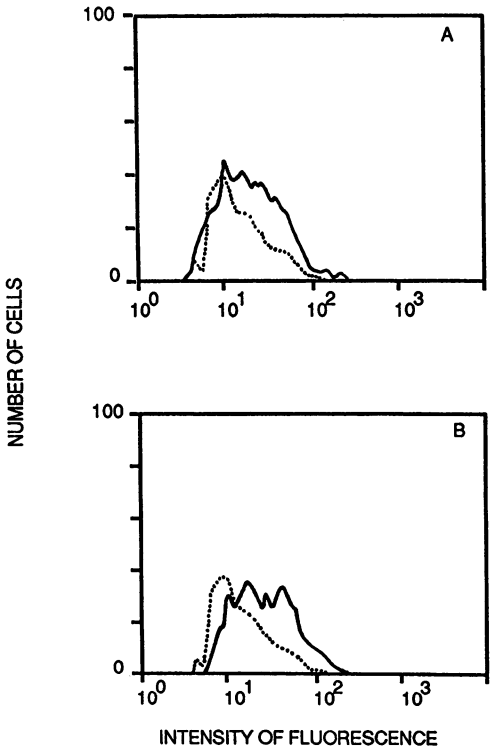

FIG. 2. Restoration of IL-2R expression by anti-IFN-γ antibodies. (A) IL-2R expression on ConA-stimulated LNC (solid line) and LNCi (broken line). (B) IL-2R expression on ConA-stimulated cocultures of LNC with LNCi in the presence (solid line) or absence (broken line) of anti-IFN-γ antibodies.

TABLE 2. Effect of in vivo treatment with anti-IFN-γ on T-cell proliferation in LNCi

Culture[a]	In vivo treatment[b]	Proliferation (Δcpm, 10^3)	% Reduction[c]
LNC	None	314 ± 10	
LNC	Anti-IFN-γ (−6 h)	322 ± 12	0
LNCi	None	113 ± 15[d]	65
LNCi	Anti-IFN-γ (−6 h)	259 ± 14[d,e]	18
LNCi	Anti-IFN-γ (+6 days)	242 ± 8[d,e]	23
SPC	None	201 ± 10	
SPCi	None	73 ± 7[d]	64
SPCi	Anti-IFN-γ (−6 h)	45 ± 9[d]	78
SPCi	Anti-IFN-γ (+6 days)	74 ± 8[d]	64

[a] LNC and LNCi cultured at a concentration of 2 × 10^6/ml were stimulated with ConA.

[b] LNC were harvested from animals that were either untreated or treated with anti-IFN-γ. LNCi were harvested from *T. brucei*-infected animals that were either untreated or treated with anti-IFN-γ antibodies (treatment 6 h before [−6 h] or 6 days after [+6 days] infection).

[c] Percent reduction was calculated relative to the proliferation of LNC of untreated, uninfected control animals.

[d] Proliferative response significantly lower than the LNC or SPC response (two-tailed Student's test, $P < 0.001$).

[e] Proliferative response significantly higher than the LNCi response (two-tailed Student's test, $P < 0.001$).

trol and anti-IFN-γ-treated groups, and no differences in the survival rates of the different experimental groups were observed. These observations indicate that the IFN-γ-mediated immunosuppression in the LNC compartment is not directly related to the control of *T. brucei* infections. This does not completely exclude a possible connection between a state of immunosuppression and parasite development since the splenic compartment remained suppressed during anti-IFN-γ treatment (Table 2).

According to the results described here, IFN-γ appears to act as a key molecule in *T. brucei*-elicited suppression of T-cell proliferative responses, at least as far as the LNC compartment is concerned. *T. brucei*-mediated induction of IFN-γ production may represent the first event in the pathway of suppression. According to another report (1), very large numbers of IFN-γ-producing $CD8^+$ T cells can be detected in *T. brucei*-infected rats as early as 12 h postinfection. This fast IFN-γ production in vivo is most probably induced directly by the parasite, since *T. brucei*-released factors were described as capable of triggering in a non-antigen-specific way a rapid release of IFN-γ by $CD8^+$ cells

TABLE 3. Effect of in vivo treatment with anti-IFN-γ on the suppressive activity of LNC from *T. brucei*-infected animals

Culture or coculture[a]	Proliferation (Δcpm, 10^3)	% Reduction[b]
LNC	420 ± 14	
LNC + LNCi	120 ± 13[c]	72
LNC + LNCi (anti-IFN-γ, −6 h)	386 ± 10	9
LNC + LNCi (anti-IFN-γ, +6 days)	380 ± 10	10

[a] LNC cultured at a concentration of 4 × 10^6/ml were stimulated with ConA. Cocultures of LNC (2 × 10^6/ml) and LNCi (2 × 10^6/ml) were made to evaluate the suppressive activity of LNCi on the response of LNC. LNCi were harvested from *T. brucei*-infected animals that were either untreated or treated with anti-IFN-γ (treatment 6 h before [−6 h] or 6 days after [+6 days] infection).

[b] Percent reduction was calculated relative to the proliferation of LNC.

[c] Proliferative response significantly lower than the LNC response (two-tailed Student's test, $P < 0.001$).

(14). Local hyperproduction of IFN-γ may in turn affect immune responsiveness through macrophage activation, for instance. In fact, experimental infections with *T. brucei* result in the activation of macrophages, as evidenced by an increase in class II major histocompatibility complex expression (8), enhanced release of reactive oxygen components (5) and prostaglandins, and increased production of IL-1 (16). Previously, we have demonstrated that LNCi contain suppressive macrophage-like cells that fully account for the inhibitory activity of LNCi populations (17). In view of the present observation that in vivo treatment with anti-IFN-γ abolishes the generation of LNCi-associated suppressive cells, it is tempting to speculate that IFN-γ is responsible for the induction of suppressive macrophages. Other reports corroborate the potential of IFN-γ to mediate an immunosuppressive activity via induction of macrophage-like suppressor cells. Cloned antigen-presenting macrophage cell lines, which initially induce immunity, can turn into cells capable of T suppressor cell induction following activation with IFN-γ (10). IFN-γ-treated macrophages were found to be inhibited in their capacity to induce antigen-specific T-cell proliferation, and the underlying mechanism of inhibition was unrelated to class II major histocompatibility complex expression, IL-1 secretion, and prostaglandin secretion (12).

Besides playing a potential role in the generation of suppressive macrophages, IFN-γ seems to be also involved directly in the inhibition of IL-2R expression and T-cell proliferation. Indeed, addition of anti-IFN-γ to cocultures of LNCi and LNC restored both the proliferative response and the expression of IL-2R. Antiproliferative activities of IFN-γ were reported for Th2 cells (4, 6), B cells (13), and bone marrow cells (7). In the experiments described above, however, the inhibition of T-cell proliferation is probably not due to an action of IFN-γ alone, since supplying 1,000 U of exogenous IFN-γ to ConA-activated LNC did not influence the mitogenic response (data not shown). Hence, the inhibitory activity of IFN-γ on IL-2R expression and T-cell proliferation requires other, ill-defined cofactors. The availability of an in vitro model that enables mimicry of the suppressive activity of *T. brucei*-elicited macrophage suppressor cells (15) will be useful in the identification of these putative suppressive cofactors.

In summary, on the basis of our accumulated data on *T. brucei*-elicited suppression in the LNC compartment, at least two soluble mediators appear to be involved in the pathway of suppression: (i) prostaglandins inhibiting IL-2 secretion (18) and (ii) IFN-γ which is involved in both the generation of suppressive cells and the prostaglandin-independent suppression of IL-2R expression. It is worth stressing that the IFN-γ-dependent pathway of suppression seems to occur only in the lymph node and not in the splenic compartment. Indeed, while anti-IFN-γ treatment restored the impaired proliferative effect of LNC, no restoration was observed in SPC populations. Recently, Sternberg and McGuigan (20) reported that nitric oxide (NO) released by macrophages mediates suppression of splenic T-cell responses in murine *T. brucei* infections. In these studies, suppression could be abrogated by N^G-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of arginine-dependent NO synthetase. We have tested the influence of L-NMMA on cocultures of LNCi and LNC and found no influence of this NO inhibitor on the suppressed response of LNC (data not shown). Hence, murine *T. brucei* infections may elicit two distinct suppressive mechanisms in different lymphoid compartments: an IFN-γ-dependent mechanism in lymph nodes and an NO-dependent mechanism in the spleen.

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