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# Different trypanozoan species possess CD8 dependent lymphocyte triggering factor-like activity

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**Abstract**

*Trypanosoma brucei brucei* (*T. b. brucei*) release a molecule, trypanosome derived lymphocyte triggering factor (TLTF), which stimulates $CD8^+$ cells to produce cytokines and to proliferate. We now report that *T. evansi*, *T. b. gambiense* and *T. b. rhodesiense* also contain factor(s) with similar activity. Thus, homogenates from these parasite taxa triggered mouse or rat lymphoid tissue of mononuclear cells (MNC) to produce interferon gamma (IFN-$\gamma$) and to proliferate. These responses were dependent on CD8 since the activity was blocked by (a) anti CD8 antibodies, (b) occurred in $CD8^+$ but not in $CD8^-$ mice and (c) was recorded in human $CD8^+$ but not in $CD4^+$ enriched peripheral blood mononuclear cells (PBL). The presence of TLTF or TLTF-like molecules in the trypanozoan species was also examined by *T. b. brucei* directed anti-TLTF Mabs using two Mabs with inhibitory activity and one with stimulatory activity. The lymphocyte triggering activity of *T. b. gambiense* and *T. b. rhodesiense*, but not *T. evansi*, was affected by the anti-TLTF Mabs. We conclude that *T. evansi*, *T. b. rhodesiense* and *T. b. gambiense* similar to *T. b. brucei*, all possess molecule(s) which CD8 dependently trigger lymphocytes. The latter three, related parasite taxa, share TLTF antibody binding epitopes.

*Keywords: Trypanosoma spp.*; CD8; IFN-$\gamma$; IL-4; TGF-$\beta$; Proliferation

## 1. Introduction

In humans, infection with *T. b. gambiense* and *T. b. rhodesiense* leads to two forms of disease, Gambian or West African sleeping sickness and Rhodesian or East African sleeping sickness, respectively. *T. b. brucei* causes trypanosomiasis in domestic animals (Nagana) but does not affect man. Another Trypanozoan species, *T. evansi*, is infective for horses, buffaloes and camels causing a disease called Surra.

*T. b. brucei* are extracellular haemoflagellates, which have developed several means to escape the immune assault of the host. They have the ability to switch to a new variant surface glycoprotein (VSG) in a subgroup of the parasite population before antibody clearance of the entire population takes place. The extreme variability of VSG has hindered vaccination attempts against this target structure and a research for a uniform molecule has become indispensable. We have described a molecule (TLTF) secreted by *T. b. brucei*, which may be an invariant molecule. TLTF binds to CD8 on lymphoid cells and triggers mononuclear lymphoid cells to IFN-$\gamma$ production and to proliferation. IFN-$\gamma$ in turn constitutes a growth stimulus for the parasite as evidenced by antibody blocking studies both in vitro and in vivo and direct growth stimulation using recombinant rat IFN-$\gamma$ [1,2]. A mouse Mab (MO1) against TLTF was raised and used to purify the molecule by affinity chromatography. Passive immunotherapy of infected rats and mice in vivo with the anti-TLTF Mab (MO1) strongly reduced parasite levels and prolonged survival of the animals [3].

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This bidirectional activation system between parasites and host lymphocytes is thus of vital importance in establishment of an active infection in the experimental host. Of greater relevance is whether such a system also operates in parasite infections of humans and economically important animals. If so, the parasite-produced molecule could be a possible target for chemotherapeutic design.

We have therefore addressed this issue by studying CD8-dependent triggering of CD8 lymphocytes to IFN-γ production after stimulation with frozen homogenates from *T. evansi*, *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. In addition, immunologically cross-reactive epitopes were sought. Hereby. MO1 and two newly raised anti-TLTF antibodies (MO2, MO3) were used in in vitro studies.

## 2. Materials and methods

### 2.1. Parasites

The following cloned monomorphic populations of the different Trypanozoan taxa were used in the present work: (a) *T. b. brucei* An Tat 2.2 (b) *T. evansi* RoTat 1.2 (c) *T. gambiense* LiTat 1.3 (d) *T. rhodesiense* ETat 1/2 R. The parasites were purified from infected mouse blood at the Laboratory of Serology, Institute of Tropical Medicine, Prins Leopold, Antwerp, Belgium according to Lanham and Godfrey [4] and washed by centrifugation in phosphate buffered saline supplemented with glucose. Pellets of pure trypanosomes were kept at −70°C. Before experimentation, the cells were disrupted by freezing and thawing.

### 2.2. Animals

Rats and mice were used in this study. Sprague-Dawley rats were purchased from Alab (Stockholm, Sweden). DBA 2 MHC H-2d/d mice were locally bred at the Department of Immunology, Karolinska Institute, Stockholm. Mutant mice ($CD8^-$) with disrupted lyt 2 gene lacking CD8 expression [5], and mutant mouse strain lacking CD4 expression [6] originally from the Department of Medical Biophysics and Immunology, University of Toronto, Canada, were bred and kept at the Department of Immunology, Karolinska Institute, Stockholm, Sweden.

### 2.3. Monoclonal antibodies

In in vitro antibody TLTF modulation experiments (see below) the following Mabs were used: (a) IB-4; a Mab directed against TNP (anti-trinitrophenylphosphate) was used as an isotype matched control antibody (mouse IgG2b). The hybridoma producing IB-4 was obtained from Dr Birgitta Heyman (Uppsala, Sweden) and antibodies were prepared from culture supernatants (b) OX8; an anti-rat CD8 antibody (mouse IgG1). The hybridoma producing OX8 was originally obtained from Dr Alan Williams (Oxford, UK). In some experiments Fab fragments of OX8 were used. The fragments were prepared by papain digestion followed by removal of Fc fragments and any undigested OX8 on protein A (c) MO1, MO2 and MO3; mouse Mabs directed against TLTF. MO1 was prepared as described previously [3], while MO2 and MO3 were prepared for this study. Ten hybridoma supernatants significantly inhibited TLTF induced IFN-γ production, while seven hybridoma supernatants exhibited stimulatory effects. One of the inhibitory hybridomas (MO2) and one of the stimulatory hybridomas (MO3) were further propagated and characterized. Both of these hybridomas produced antibodies of the IgG2b subclass and were purified on Protein A Sepharose according to standard techniques.

### 2.4. Single cell assay for IFN-γ secretion

The method described by Czerkinsky [7] as adapted to rat IFN-γ [8] was used to detect IFN-γ production by single secretory cells [3].

### 2.5. Lymphocyte proliferation assay

Aliquots of $5 \times 10^6$ MNC per ml [2] were added in triplicate to 96-round-bottomed well microtitre plates (Nunc, Copenhagen, Denmark). The cells were incubated 72 h. Ten hours before harvest 10 μl aliquots containing 1 μCi of [$^3$H]-methylthymidine (specific activity 42 Ci/mmol) (Amersham, Little Chalfont, UK) in saline were added to each well. Cells were harvested onto glass fibre filters with a multiple channel semiautomated harvesting device (Titertek, Skatron AS, Lierbyen, Norway) and thymidine incorporation was measured as counts/min in a liquid beta-scintillation counter (Mark II, Searle, Analytic, Des Plaines, IL).

### 2.6. In situ hybridization for detection of IFN-γ, IL-4 and TGF-β mRNA

The method described by Dagerlind [9] was used to study ctyokine mRNA expression as shown previously [2]. The oligonucleotide sequence for IFN-γ, TGF-β and IL-4 was obtained from GenBank through the use of the MacVector system.

### 2.7. Antibody modulation experiments

Ten-microlitre aliquots of the IB-4, OX8, MO1, MO2 and MO3 Mabs to obtain final concentration in medium of 5 μg/ml were added to microtitre plate wells immediately after plating the MNC. Ten-microlitre

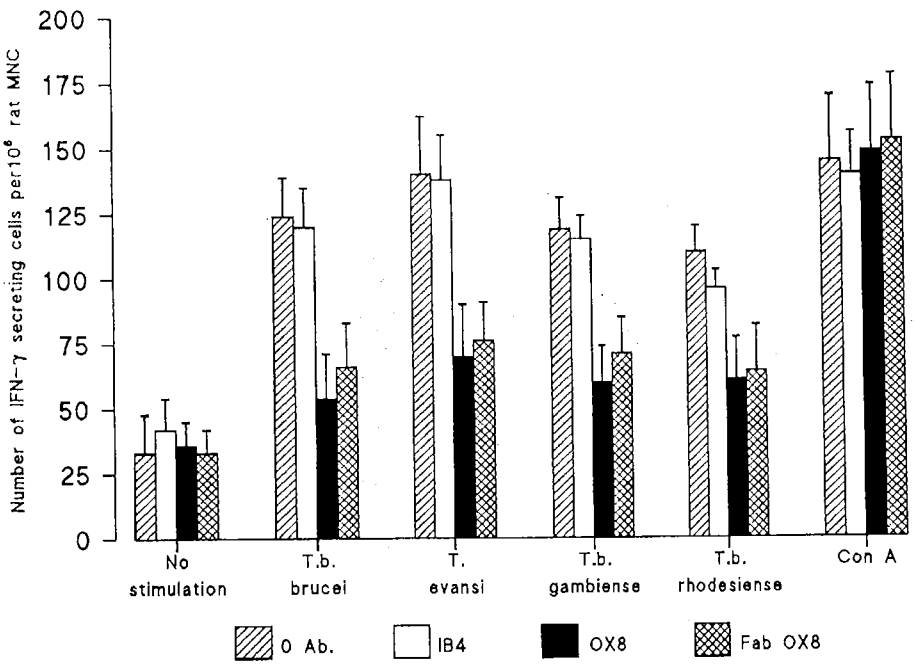


Fig. 1. Monoclonal antibody inhibition of normal rat spleen cell triggering by Con A and homogenates of the different trypanosome strains. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h. The number of IFN-$\gamma$ secreting cells was measured using the immunospot assay. Immediately before addition of optimal dilutions of Con A or homogenates from different parasite strains, different cultures received whole mouse anti rat CD8 (OX8) monoclonal antibody (filled staples) or its Fab fragment (cross-hatched staples) to reach a final concentration in the medium of 5 $\mu$g/ml. An isotype cross-matched control antibody (IB4) at a similar final concentration was added to additional cultures (open staples). Other cultures (0) received no antibodies. The specificities of the antibodies are described in the materials and methods. Each staple denotes data from nine cultures. Bars denote S.D.

aliquots of frozen and thawed trypanosomes were added. As a control, 10 $\mu$l of Con A (5 $\mu$g/ml as final concentration in medium; Pharmacia, Uppsala, Sweden) was used. After 24 h and 72 h of culture the effects on IFN-$\gamma$ secretion and cell proliferation were determined, respectively.

### 2.8. Statistics

Mann-Whitney's test was used for statistical significance.

## 3. Results

### 3.1. Anti CD8 antibody blocking studies

We here reproduce our previous finding that exposure of rat spleen MNC to a frozen and thawed preparation of *T. b. brucei* for 24 h caused a striking increase in the number of IFN-$\gamma$ secreting cells (SC), and that this phenomenon was dependent on CD8 [1,2]. Frozen and thawed preparations of the other trypanosomes similarly triggered rat MNC to IFN-$\gamma$ production (Fig. 1). The number of cells induced to IFN-$\gamma$ secretion by the different parasite preparations amounted to approximately 125 IFN-$\gamma$ SC per $10^6$ MNC, which is in the same range of that obtained after Con A stimulation of parallel cultures. Mouse Mab against rat CD8 was evaluated for its capacity to reduce the action of frozen trypanosomes on rat splenocyte MNC. Intact anti-CD8 Mab or its Fab fragment strongly reduced IFN-$\gamma$ production induced by all parasite taxa, while Con A stimulated IFN-$\gamma$ production remained unaffected. Control antibody had no effect (Fig. 1).

### 3.2. Studies on cells from mice with $CD8^-$ and $CD4^-$ genotype

Cells from mutant mice lacking CD8 or CD4 expression were used to further test the role of $CD8^+$ T cells in the MNC triggering by the different parasite strains. Exposure of spleen MNC obtained from the $CD8^-$ mice in vitro to parasite homogenates caused no proliferation or increased cytokine production as evaluated by the number of IFN-$\gamma$ secreting cells and expression of IFN-$\gamma$, IL-4 and TGF-$\beta$ mRNAs was recorded. Conversely, a notable proliferative response and high levels of cells expressing mRNA for IFN-$\gamma$, TGF-$\beta$ after exposure to the different parasite strains was recorded in MNC from $CD4^-$ mice. There was also a slight decrease in numbers of cells expressing mRNA for IL-4. The responses in the CD4 mice to parasite homogenate triggering were comparable to those ob-

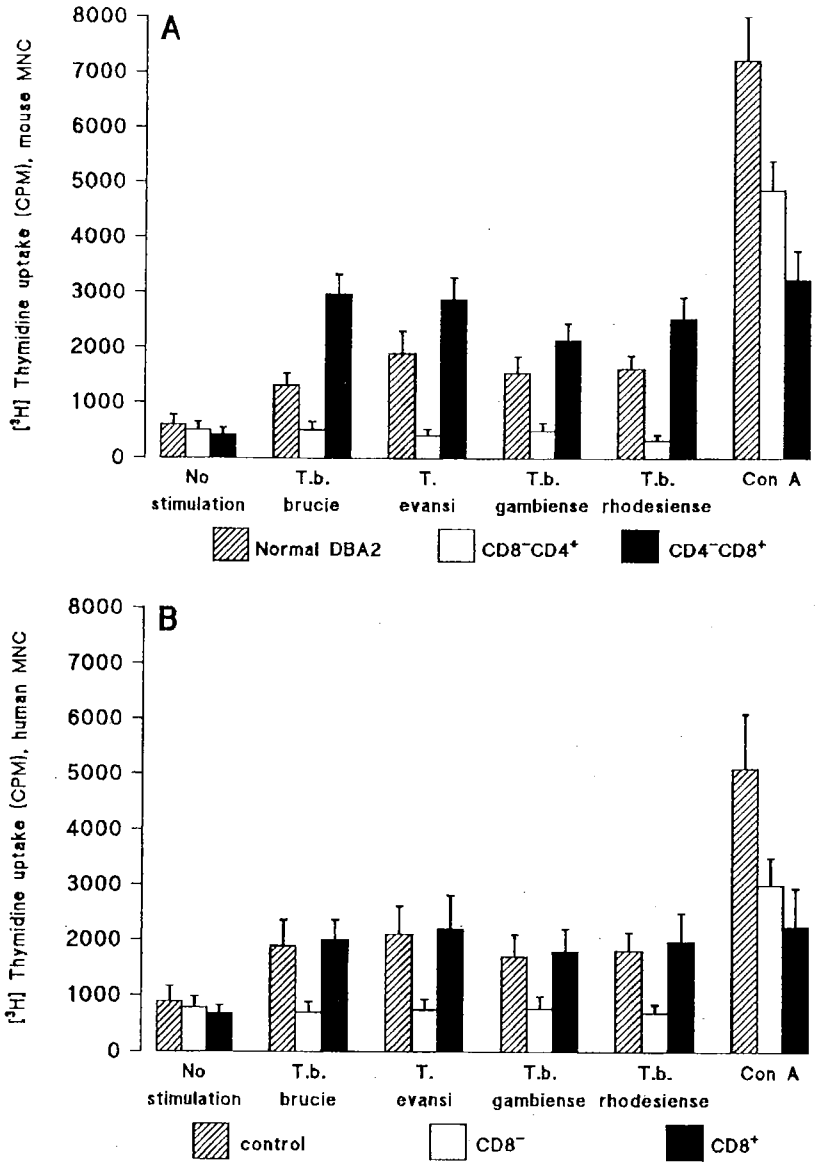


Fig. 2. Proliferation effect of homogenates from different parasite strains and Con A on mouse spleen MNC from genomic CD4$^-$ mice (containing CD8$^-$ cells), CD8$^-$ mice (containing CD4$^+$ cells) or normal DBA2 (A), and on whole human peripheral blood mononuclear cells (PBL), purified CD8$^+$ and CD4$^+$ T cells (B) as assessed by $^3$H-thymidine uptake. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 72 h. Triplicate cultures were exposed to optimal dilutions of homogenates from different parasite strains or Con A. Control cultures received no stimulation. Each staple represents data from triplicate cultures. Bars denote S.D. The experiment was repeated twice with similar results.

tained in the normal DBA2 mice. Con A induced the production of all three cytokines studied in these mice (Fig. 2A, Fig. 3A, Fig. 4A, Fig. 5A, Fig. 6A).

### 3.3. Effects on purified human CD4$^+$ and CD8$^+$ T cells

Human CD4$^+$ and CD8$^+$ T cell enriched MNC populations [2] were exposed to Con A, which induced T cell triggering assessed by proliferation, number of IFN-$\gamma$ SC, or number of mRNA expressing cells for IFN-$\gamma$, IL-4 and TGF-$\beta$. However, these responses were somewhat reduced for both CD4$^+$ cells and CD8$^+$ T cells, compared to whole PBL. In contrast, all trypanosome homogenates induced a proliferative response and high levels of cells expressing mRNA for IFN-$\gamma$, TGF-$\beta$ and to a lower extent mRNA for IL-4 production among CD8$^+$ T cells alone. These responses were also comparable in magnitude to those among whole PBL, but were absent among CD4$^+$ T cells (Fig. 2B, Fig. 3B, Fig. 4B, Fig. 5B, Fig. 6B).

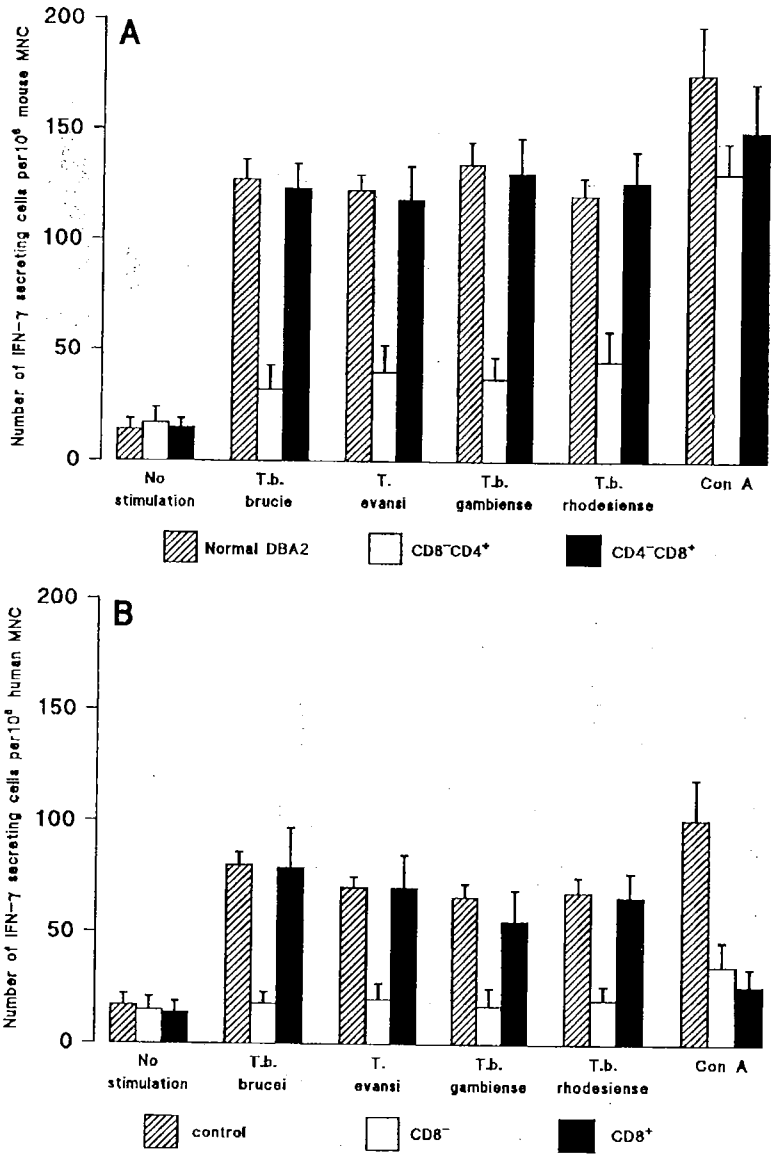


Fig. 3. Effects of homogenates from different parasite strains and Con A on mouse spleen MNC from genomic CD4$^-$ mice (containing CD8$^+$ cells), CD8$^-$ mice (containing CD4$^+$ cells) or normal DBA2 (A), and on whole human peripheral blood mononuclear cells (PBL), purified CD8$^+$ and CD4$^+$ T cells (B) as assessed by the number of IFN-$\gamma$ secreting cells determined in the immunospot assay. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h. Triplicate cultures were exposed to optimal dilutions of homogenates from different parasite strains or Con A. Control cultures received no stimulation. Similar data were obtained on three different occasions. Bars denote S.D.

### *3.4. Monoclonal antibody modulating effects on IFN-γ secretion of rat MNC triggered by different parasite strain homogenates*

Addition of the mouse monoclonal anti *T. b. brucei* TLTF antibodies, MO1 and MO2, at the start of rat MNC culture strongly reduced the IFN-$\gamma$ secretion induced by frozen and thawed *T. b. brucei* and *T. b. rhodesiense* (Fig. 7A,B). MO3, the third anti-TLTF antibody which enhanced the triggering to IFN-$\gamma$ secretion by *T. b. brucei* markedly increased the number of IFN-$\gamma$ producing cells when incubated with MNC cultures stimulated with frozen *T. b. rhodesiense* and *T. b. gambiense* (Fig. 7B). None of the anti-TLTF Mabs affected the number of IFN-$\gamma$ producing cells after stimulation with *T. evansi*. IB-4, which was used as an isotype-matched control antibody did not affect the number of IFN-$\gamma$ SC induced by any strain (Fig. 7A). Neither the anti-TLTF Mabs nor the control antibody had any effect on Con A induced IFN-$\gamma$ secretion (Fig. 7A,B).

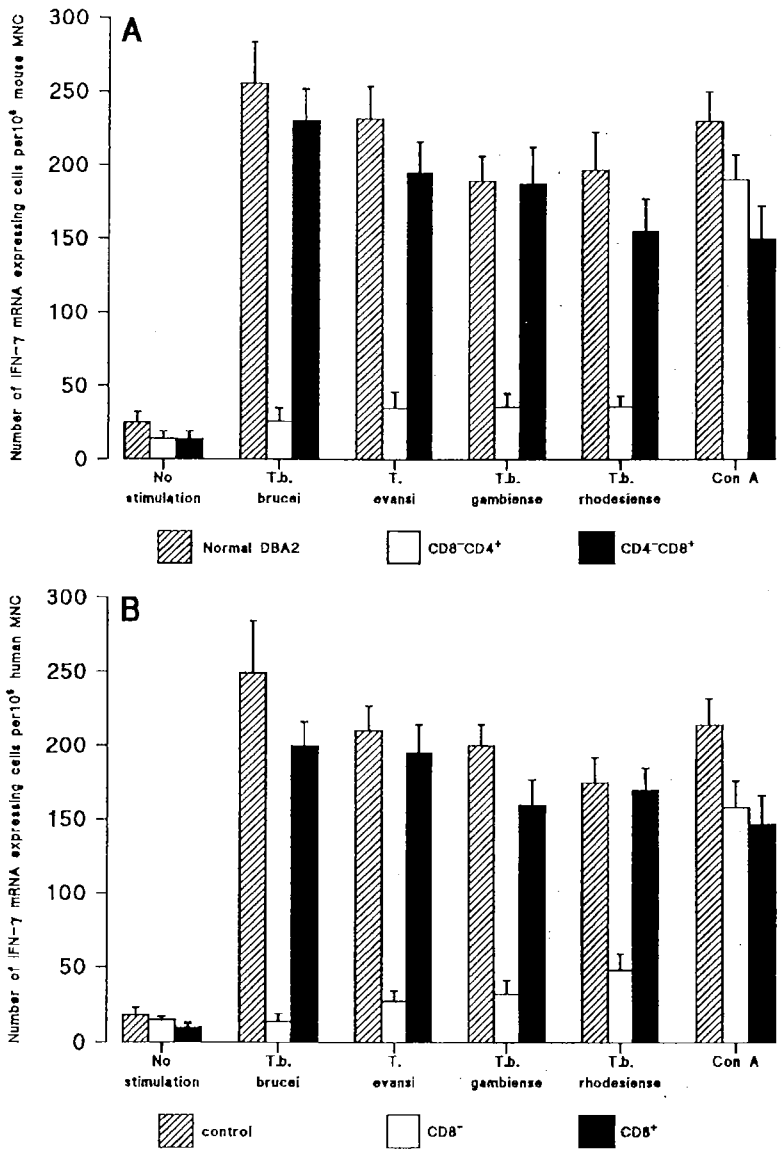


Fig. 4. Effects of homogenates from different parasite strains and Con A on mouse spleen MNC from genomic $CD4^-$ mice (containing $CD8^+$ cells), $CD8^-$ mice (containing $CD4^+$ cells) or normal DBA2 (A), and on whole human peripheral blood mononuclear cells (PBL), purified $CD8^+$ and $CD4^+$ T cells (B) as assessed by the number of cells expressing mRNA for IFN-γ. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h after exposure to optimal dilutions of homogenates from different parasite strains or Con A. Control cultures received no stimulation. Data are expressed as number of mRNA expressing cells per $10^5$ MNC. Similar data were obtained on two different occasions. Bars denote S.D.

## 4. Discussion

We have demonstrated that frozen and thawed homogenates of *T. evansi*, *T. b. rhodesiense* and *T. b. gambiense* all caused a triggering of lymphocytes dependent on presence of $CD8^+$ cells. This $CD8^+$ cell dependency was evidenced by three types of experiments; (i) a mouse monoclonal anti rat CD8 antibody or its Fab fragment reduced triggering of rat MNC, (ii) triggering did not occur in genomically CD8 deleted mouse cells, but was conspicuous in CD4 deleted or normal mouse cells (iii) human $CD8^+$, but not $CD4^+$ enriched cells responded. Thus, these parasites contain one or more molecules which act on $CD8^+$, similar to what we have observed for *T. b. brucei* [1]. These observations are partly consistent with those of Darji [10], who also reported a conspicuous $CD8^+$ cell triggering during experimental African trypanosomiasis. The reduction of cell activation with anti CD8 antibodies and their Fab fragments suggests that the actual target for

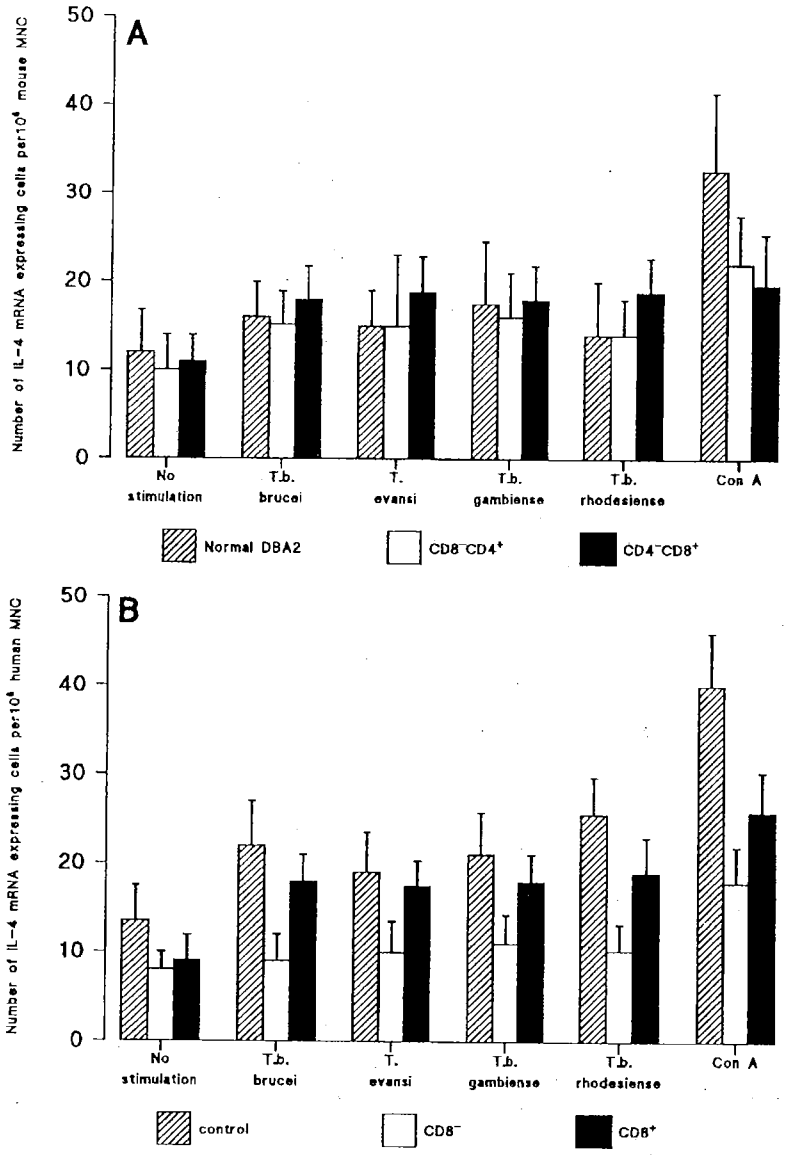


Fig. 5. Effects of homogenates from different parasite strains and Con A on mouse spleen MNC from genomic $CD4^-$ mice (containing $CD8^+$ cells), $CD8^-$ mice (containing $CD4^+$ cells) or normal DBA2 (A), and on whole human peripheral blood mononuclear cells (PBL), purified $CD8^+$ and $CD4^+$ T cells (B) as assessed by the number of cells expressing mRNA for IL-4. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h after exposure to optimal dilutions of homogenates from different parasite strains or Con A. Control cultures received no stimulation. Data are expressed as number of mRNA expressing cells per $10^5$ MNC. Similar data were obtained on two different occasions. Bars denote S.D.

cell activation is the CD8 molecule itself. It remains to be determined if other cell surface structures on $CD8^+$ cells and/or accessory cells are also involved.

Thus there appears to exist a conserved effect of CD8 activation among different African trypanosome species. There is a possibility that the different parasite species achieve this activation by different processes, but our results suggest a common mechanism. More detailed information as to the structural variability of the CD8 activating molecules between species is required, but some evidence for disparity in composition was evidenced by our antibody inhibition studies. To this end, we examined if the $CD8^+$ activating components from the different trypanosome strains showed antibody binding epitopes in common with TLTF, as defined and obtained from *T. b. brucei*. Such sharing of epitopes would suggest structural homology between the strains. Ideally, such comparisons should be made after sequencing of TLTF, whereby corresponding molecules can more easily be sought in the other

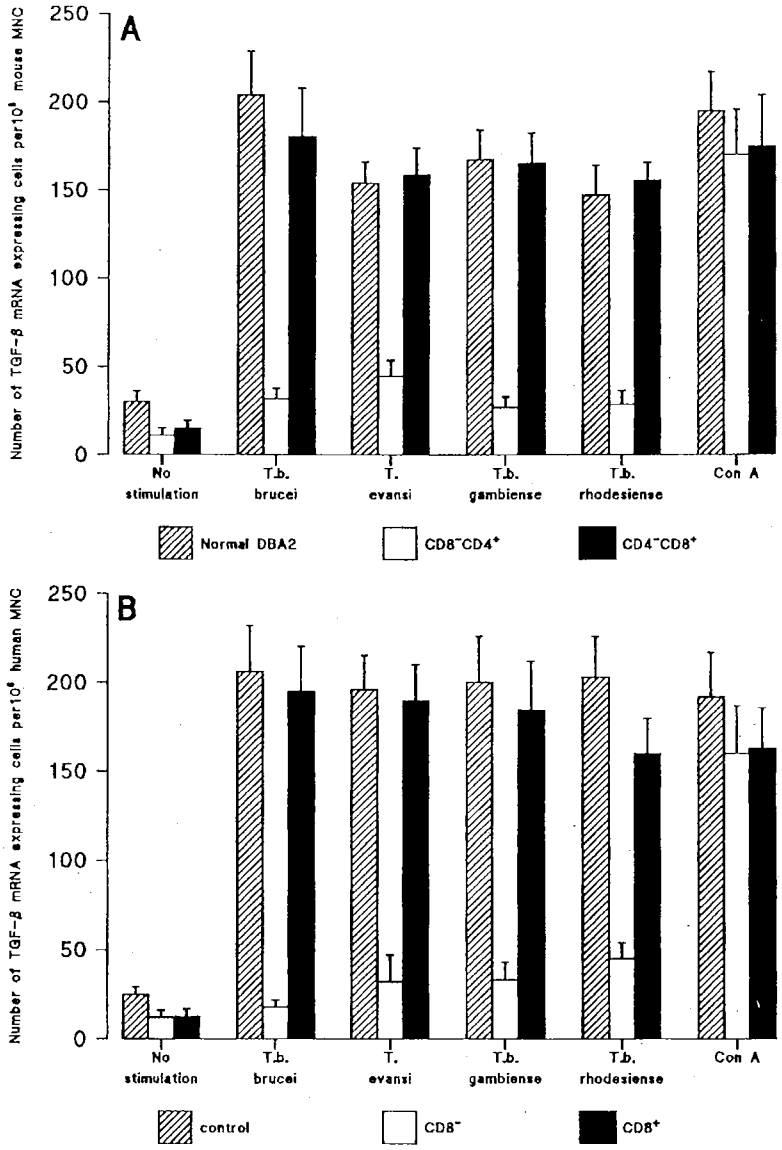


Fig. 6. Effects of homogenates from different parasite strains and Con A on mouse spleen MNC from genomic CD4$^-$ mice (containing CD8$^-$ cells), CD8$^+$ mice (containing CD4$^-$ cells) or normal DBA2 (A), and on whole human peripheral blood mononuclear cells (PBL), purified CD8$^-$ and CD4$^+$ T cells (B) as assessed by the number of cells expressing mRNA for TGF-$\beta$. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h after exposure to optimal dilutions of homogenates from different parasite strains or Con A. Control cultures received no stimulation. Data are expressed as number of mRNA expressing cells per $10^5$ MNC. Similar data were obtained on two different occasions. Bars denote S.D.

strains. At this stage, however, we could study antibody modulation of the IFN-$\gamma$ responses induced by the different parasite strains. Hereby, effects of one or more of the antibodies primarily raised against *T. b. brucei* TLTF were noted on the responses induced by putative TLTFs from *T. b. rhodesiense* and *T. b. gambiense*, but not on the responses induced by *T. evansi*. This suggests that the CD8$^+$ activating material in the former strains is more closely related to *T. b. brucei* TLTF than material from the latter. Furthermore, only MO3 affected *T. b. gambiense*, while all three antibodies affected in part *T. b. rhodesiense*, suggesting that CD8$^-$ activating material from the latter strain has more antibody binding epitopes in common with *T. b. brucei* TLTF than the former.

The profile of cytokines induced during CD8$^+$ cell dependent triggering was similar with the different parasite strains, with a conspicuous triggering of IFN-$\gamma$, TGF-$\beta$ and to a low extent IL-4. A series of other important cytokines and their interplay remains to be

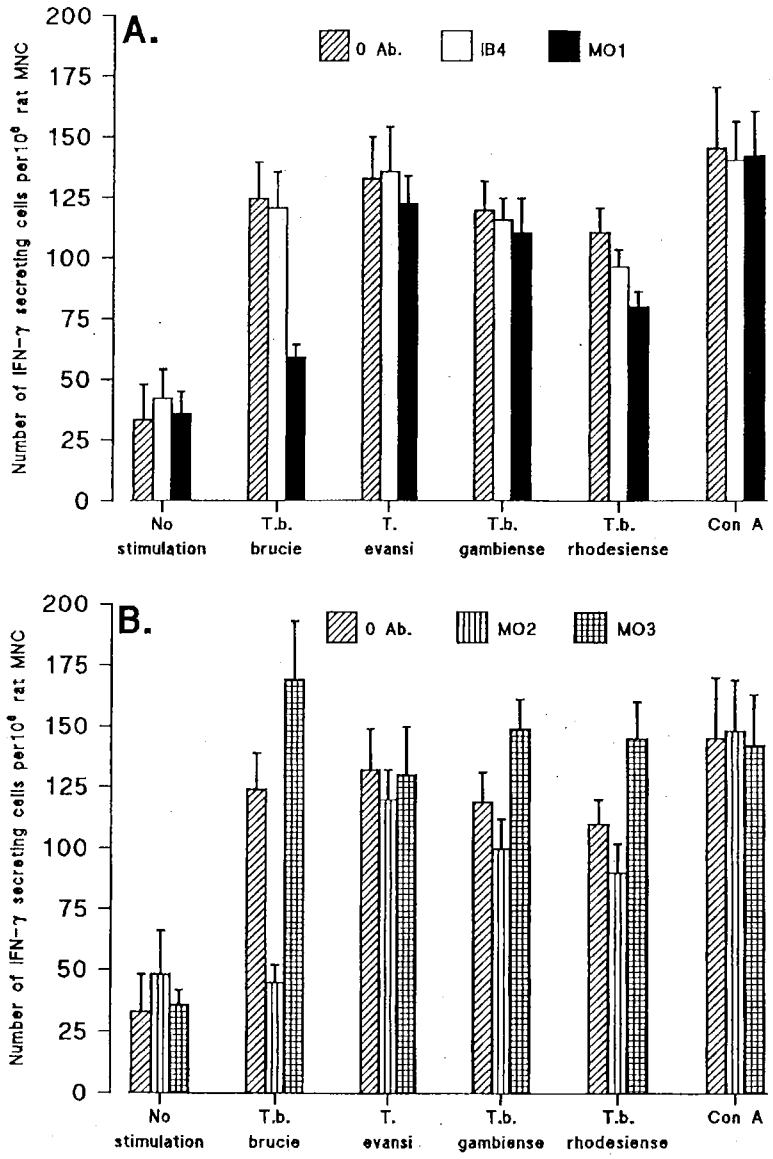


Fig. 7. The effect of mouse anti-*T. b. brucei* TLTF monoclonal antibodies on the triggering of normal rat spleen cells by the different parasite homogenates. Cell suspensions at a density of $10^6$ cells per well were plated and cultured for 24 h. The number of IFN-$\gamma$ secreting cells was measured by the immunospot assay. Immediately before addition of optimal dilutions of Con A or homogenates from the different parasite strains, different cultures received whole mouse anti *T. b. brucei* TLTF monoclonal antibodies MO1 (A); MO2 (B) or MO3 (B) to reach a final concentration in the medium of 5 $\mu$g/ml. An isotype cross-matched control antibody (IB4) at a similar final concentration was added to additional cultures (A). Other cultures (left hatched staples in A and B) received no antibodies. Each staple denotes data from nine cultures. Bars denote S.D.

studied in this context. It is of interest to note that production of both TNF-$\alpha$ and IL-1$\alpha$ depend on IFN-$\gamma$, since IFN-$\gamma$ receptor deleted mice fail to produce these cytokines upon challenge [11]. IFN-$\gamma$ seems to have a special role, at least in experimental infection with *T. b. brucei*, since it may provide a direct growth stimulus for the parasite, as evidenced by (i) blocking of lymphoid feeder cell supported growth of the parasite by mono- or polyclonal antibodies against IFN-$\gamma$ in vitro [1], (ii) direct growth stimulation of the parasite with recombinant rat IFN-$\gamma$ [1,3] or with a neuronal IFN-$\gamma$ like molecule [12,13], (iii) reduction of lately occurring parasite levels [2] as well as reduction of first wave parasitemia in mice [14] by in vivo treatment with anti IFN-$\gamma$ antibody. IFN-$\gamma$ may in part also be responsible for the immunosuppression evolving during experimental infection [14].

The increased IFN-$\gamma$ production during *T. b. brucei* infection may also benefit parasite survival by affecting the balance of cytokines produced by TH1/TH2 cells

[15]. Preferential recruitment and activation of TH1 cells producing IFN-γ often suppresses the activation and recruitment of TH2 cells [15,16]. Thus it is highly likely that during establishment of our experimental infection, during the phase in which there is rapid parasite growth, high levels of TLTF-induced $CD8^-$ IFN-γ production suggest a dominant TH1-like response. Production of IFN-γ by $CD4^+$ lymphocytes has also been reported in response to VSG [17].

TGF-β is of interest due to its role as an endogenous immunosuppressive molecule, elegantly demonstrated in mice with genomic deletion of TGF-β, developing spontaneous widespread inflammation [18,19]. As regards to IL-4 induction, this occurred to a low extent in mouse and human cells. We have found that the level of production of this cytokine, in contrast to IFN-γ, varies considerably between strains of mice (M. Bakhiet, L. Jansson, P. Büscher, R. Holmdahl, K. Kristensson and T. Olsson, unpublished data).

The cognate pattern of cytokines elicited by the different parasite strains, however, suggests that they may use similar survival strategies in the mammalian host. Given that the activation mechanisms were demonstrated to be similar in different parasite strains, further characterization and comparison of the nature of the activating molecules is a necessary undertaking.

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