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African trypanosome infections in mice that lack the interferon-γ receptor gene: nitric oxide-dependent and -independent suppression of T-cell proliferative responses and the development of anaemia

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SUMMARY

Infection of mice with African trypanosomes leads to a severe immunosuppression, mediated by suppressor macrophages. Using *ex vivo* macrophage culture and *in vivo* cell transfer, it has been shown that nitric oxide (NO) is a potent effector product of these cells and causes both lymphocyte unresponsiveness and dyserythropoiesis. We explored the role of NO *in vivo* during trypanosome infection using mice with a disrupted interferon-γ-receptor gene, which were unable to respond with macrophage activation and NO synthesis. These mice were less effective at controlling parasitaemia than the wild types, but showed an improved splenic T-cell responsiveness and reduced anaemia during the early stages of infection. The data indicate that, in the mouse, NO is a significant mediator of immunosuppression only in early infection. Beyond day 10 of infection, NO-independent mechanisms are of primary significance and the control of parasitaemia and T-cell responsiveness are not directly related.

INTRODUCTION

Infection with African trypanosomes (*Trypanosoma brucei sp.*) is a major cause of disease in humans and livestock in Sub-Saharan Africa. These bloodstream-dwelling protozoan parasites must establish chronic infection in their mammalian hosts in order to maintain transmission via the Tsetse fly vector, and two independent strategies for the evasion of the immune system have been described. One of these is antigenic variation, which has been the target of extensive molecular study.[1] The second is a less well-defined set of mechanisms that lead to severe immune dysfunction. Immune suppression is a commonly observed consequence of African trypanosome infections in humans,[2] domestic animals[3] and experimental rodent hosts.[4] One feature of this condition is the abolition of T and B-cell proliferative responses to both trypanosomal and heterologous antigens,[4] a process that involves suppressor macrophages[5] which possess the characteristics of activated macrophages.[6] In the early phase of murine *T. brucei* infection (up to 14 days), two products of activated macrophages, nitric oxide (NO)[7,8] and prostaglandins (PGs)[8] have been shown to mediate immunosuppressive effects *in vitro* experiments. During *T. brucei* infection, high levels of NO synthesis occur in peritoneal, splenic and bone marrow macrophages. Bone marrow macrophage NO synthesis is also a contributory factor in the anaemia associated with trypanosomiasis.[9] While *T. brucei* is susceptible to NO-mediated killing *in vitro*[10] haemoglobin acts as an NO sink and thus these parasites are protected from NO in the bloodstream environment.[11] The contribution of NO to pathogenesis in *T. brucei* infections *in vivo* was revealed through the systemic inhibition of NO synthase (NOS) using chemical inhibitors. Under these conditions there was an improved control of the first wave of parasitaemia[12] and a restoration of lymphoproliferative responses. Thus, it appears that in African trypanosome infection, we are presented with a reversal of the widely applicable paradigm of NO as an antimicrobial effector.

Macrophage activation and the resultant expression of inducible NO synthase (iNOS) is triggered by interferon-γ (IFN-γ) and as yet unidentified parasite factors.[13] Elevated IFN-γ is a characteristic feature of trypanosomiasis and appears to derive from multiple sources including $CD4^+$ T-cells,[14] $CD8^+$ T-cells[15] and natural killer (NK) cells.[16] As IFN-γ is a necessary co-activator of macrophages in trypanosomiasis we predicted that mice defective for the IFN-γ receptor as a result of homozygous gene disruption (IFN-$\gamma R^{-/-}$)[17] would show no macrophage NO synthesis during *T. brucei* infection and would therefore provide a model in which the role of NO in murine trypanosomiasis might be further explored. In this report we describe the course of parasitaemia, NO synthesis, T-cell responsiveness and anaemia in trypanosome infections in IFN-$\gamma R^{-/-}$ mice.

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MATERIALS AND METHODS

Trypanosomes and mice

Trypanosoma brucei rhodesiense EATRO2340[18] was used throughout this study. Female 129 mice, homozygous for a

disruption of the IFN-γ receptor gene and wild types,[19] were maintained in specific pathogen-free facilities. Infections were initiated with 5×10^4 parasites, injected intraperitoneally (i.p.), in groups of 13 mice, of which five were monitored daily by tail venesection. Parasitaemia was determined by adding 5μl of blood to 45 μl 0·85% NH_4Cl to lyse erythrocytes, followed by haemocytometer counting. The limit of detection was 1×10^5/ml. In addition, erythrocyte concentration was measured and a sample of serum was prepared for nitrate analysis. The remaining eight mice in each group were killed on days 3, 5, 7 and 9 postinfection (two mice on each day) for *ex vivo* assays. All data presented are representative of two independent infection studies.

Splenocyte preparation and proliferation assay

Spleens were removed aseptically, the capsule disrupted with a loose fitting teflon homogenizer and erythrocytes hypotonically lysed, as described previously.[20] Splenocytes were cultured in quadruplicate at 5×10^5 cells/flat-bottom microtitre plate well in 0·2 ml RPMI-1640 (Life Technologies, Paisley, UK), 2 mM L-glutamine and 5% heat-inactivated donor horse serum (Sigma, Poole, UK) in a 5% CO_2 in air atmosphere. In appropriate groups, the iNOS inhibitor N^G-methyl-L-arginine (L-NMMA, Calbiochem, La Jolla, CA) was included at 0·5 mM.[7] For nitrite assay and enzyme-linked immunosorbent assay (ELISA), cultures were incubated for 48 hr and then supernatants were aspirated. IFN-γ was determined in appropriate supernatants using an ELISA based on the capture antibody R4-6A2 and detection antibody XMG1·2 (Pharmigen, San Diego, CA), the assay being carried out according to the supplier's protocol. In proliferation assays, Concanavalin A (Con A, Boehringer, Lewes, UK) was included at a final concentration of 10 μg/ml. After 48 hr incubation, the cells were pulsed for 16 hr with [^{3}H]thymidine (Amersham, Bucks, UK; 25 Ci/mmol; 0·5 μCi/well). Thymidine incorporation was determined by cell harvesting and liquid scintillation counting.

Nitrite and nitrite assays

Nitrite accumulation in cell culture supernatants was determined by adding an equal volume of the Griess reagent (0.5% sulphanilamide, 0.05% naphthylethylenediamine in 2·5% H_3PO_4) followed by measurement of A_{550} as described previously.[7] For bloodstream nitrate analysis, serum was prepared at each tail venesection and diluted 1:5 in phosphate-buffered saline (PBS). Nitrate was then reduced to nitrite using *Aspergillus* nitrate reductase (Sigma) as described previously,[11] and nitrite was determined using the Griess assay as described above.

RESULTS

T. brucei infection profile in IFN-γR$^{-/-}$ mice

As IFN-γ plays an essential co-activating role in macrophage iNOS induction,[21] we predicted that IFN-γR$^{-/-}$ mice would provide a model to examine the role of NO in the control of parasitaemia in *T. brucei* infection. Following infection of IFN-γR$^{-/-}$ and wild-type mice, trypanosomes were first detected in the bloodstream at day 3 postinfection (Fig. 1a). During the first parasitaemic wave (days 3–10 postinfection) the peak parasitaemia in IFN-γR$^{-/-}$ mice was greater than

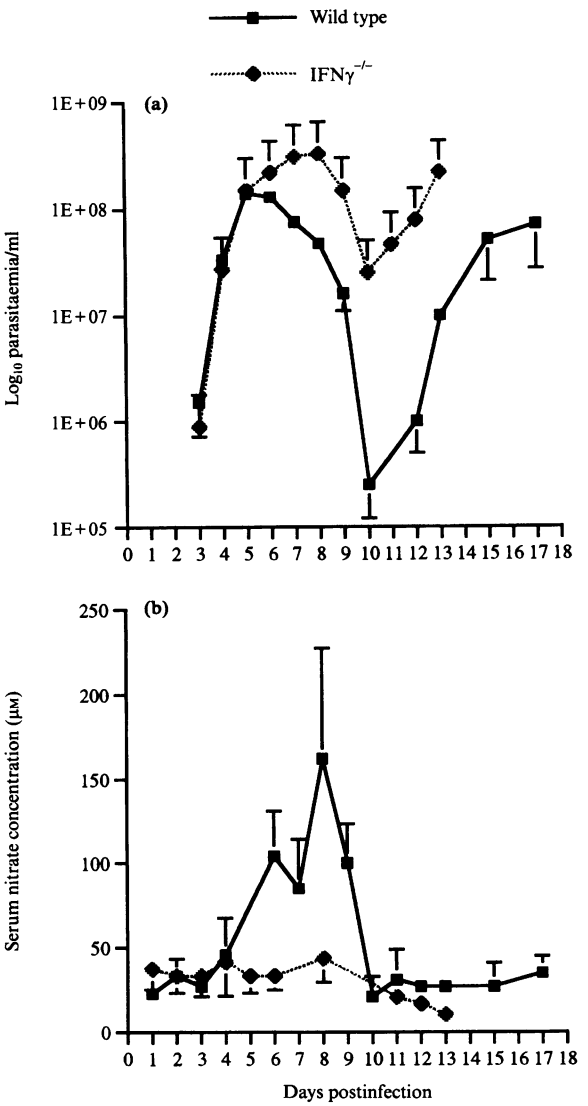

Figure 1. Parasitemia profile (a) and serum nitrate concentration (b) in 129 wild-type and IFN-γR$^{-/-}$ mice following infection with *T. brucei rhodesiense* EATRO2340. Data are presented as mean ± SE ($n=5$), and are representative of two independent infection studies.

twofold higher than that in the controls (IFN-γR$^{-/-}$:$3{\cdot}2 \times 10^8$/ml, wild type: $1{\cdot}4 \times 10^8$/ml; $P<0{\cdot}01$, paired *t*-test). For the remainder of the infections, the IFN-γR$^{-/-}$ mice continued to exhibit significantly higher parasitaemia ($P<0{\cdot}001$, repeated measure ANOVA) through a trough at day 10 post infection and into a second parasitaemic wave. During the second parasitaemic wave all the IFN-γR$^{-/-}$ mice died between days 13 and 14, whereas all the wild-type mice survived to day 17, at which point the experiment was terminated.

NO synthase activity during infection

Systemic NO synthesis was determined from plasma nitrate concentrations, this being a stable product of oxidation of NO *in vivo*.[22] In *T. brucei*-infected IFN-γR$^{-/-}$ mice, there was no increase in plasma nitrate beyond basal levels, observed in

uninfected mice, which derive from dietary nitrates (Fig. 1b). In the wild-type mice, serum nitrate levels increased with rising parasitaemia, reaching a peak of 161 μM at day 8 of infection, but returning to uninfected control levels by day 10 of infection. The peak serum nitrate level lagged approximately 3 days behind the first parasitaemic peak. The decline in serum nitrate on day 10 of infection coincided with the end of the first parasitaemic wave. However, although parasitaemia rose again from day 10 to day 17, there was no significant increase in serum nitrate during this phase of the infection. The absence of NO synthase induction in the infected IFN-γR$^{-/-}$ mice was confirmed by measurements of nitrite accumulation in *ex vivo* spleen cell cultures (Table 1). In splenocyte cultures from IFN-γR$^{-/-}$ infected mice there was no increase in nitrite concentration over background, while in splenocyte cultures derived from infected wild-type mice there were significant levels of nitrite produced on days 5, 7 and 9 of infection. Similar data were obtained with *ex vivo* peritoneal cell cultures (data not shown). IFN-γ production was measured in splenocyte cultures to confirm that the lack of NO production in the IFN-γR$^{-/-}$ mice was not a result of reduced levels of IFN-γ. In both wild-type and IFN-γR$^{-/-}$ mice, spleen cells produced similar and significantly increased amounts of IFN-γ, following trypanosome infection, in comparison with naive animals (Table 2).

These results confirm that the production of NO in response to *T. brucei* infection requires a functional IFN-γ receptor and indicate that in wild-type mice, NO synthesis is primarily associated with the first parasitaemic wave.

Table 1. Nitrite accumulation (μM) ± SD ($n=6$) in 48-hr spleen cell culture supernatants from *T. brucei*-infected and naive wild-type and IFN-γR$^{-/-}$ mice

Day of infection	Wild type		IFN-γR$^{-/-}$	
	Naive	Infected	Naive	Infected
Day 3	0·09 ± 0·09	0·18 ± 0·18	0·0 ± 0·09	0·27 ± 0·18
Day 5	0·45 ± 0·27	17·05 ± 0·71**	0·18 ± 0·27	0·36 ± 0·09
Day 7	0·0 ± 0·18	8·04 ± 0·16**	0·45 ± 0·45	0·45 ± 0·27
Day 9	0·18 ± 0·09	1·7 ± 0·27*	0·36 ± 0·27	0·36 ± 0·27

*$P<0·05$, **$P<0·001$; significantly increased over IFN-γR$^{-/-}$ infected.

Table 2. Interferon-γ production *ex vivo* by spleen cells from naive and *T. brucei*-infected wild-type and IFN-γR$^{-/-}$ mutant mice

Day post infection	Interferon concentration*			
	Wild-type mice		IFN-γR$^{-/-}$ mice	
	Naive	Infected	Naive	Infected
2	bdl	101·2	bdl	105·9
4	bdl	93·3	bdl	101·3
6	bdl	69·7	bdl	70·0

*Spleen cells were cultured for 48 hr at 37°. The concentration of IFN-γ is expressed as units/5 × 10^5 cells. All SD values were less than 5% of the mean. bdl, below limit of detection (10 U/ml).

Role of NO in the suppression of T-cell proliferative responses

As macrophages from *T. brucei*-infected IFN-γR$^{-/-}$ mice did not produce NO, we studied the inhibition of the Con A proliferative response in these and also in the wild-type mice during infection in order to determine the involvement of NO in suppression. In wild-type mice, there was a dramatic inhibition of T-cell proliferation in response to Con A from day 5 of infection (Table 3), which was partially abrogated by the inhibitor of iNOS, L-NMMA. However, the degree of restoration of the proliferative response in the presence of L-NMMA diminished from seven fold on day 5 of infection to two-fold on day 9, suggesting that as the infection progressed, the contribution of NO to suppression became less significant. This interpretation is completely consistent with the Con A responses observed in the IFN-γR$^{-/-}$ mice. In these animals, while T-cell proliferative responses to Con A are also suppressed from day 5, the degree of suppression is reduced compared with that of the infected wild types. Addition of L-NMMA to splenocyte cultures from infected IFN-γR$^{-/-}$ mice did not further restore proliferative responses.

The onset of anaemia in IFN-γR$^{-/-}$ mice

Erythrocyte density in IFN-γR$^{-/-}$ and wild-type infected mice is presented in Fig. 2. Despite higher parasitaemia, the IFN-γR$^{-/-}$ mice showed significantly reduced anaemia during the first parasitaemic wave ($P<0·001$, repeated measures ANOVA). This distinction disappeared at day 10 postinfection, coinciding with the partial clearance of the first parasitaemic wave. During the second parasitaemic wave the IFN-γR$^{-/-}$ mice exhibited a significantly more severe reduction in red cell density than the wild types at the same duration of infection.

DISCUSSION

Previous studies have identified NO as an effector of immunosuppression in murine trypanosomiasis.[16] As IFN-γ synergizes with parasite-derived macrophage activating factors in the induction of iNOS,[13] we analysed the course of *T. brucei* infection in mice lacking a functional IFN-γ receptor. Following infection with *T. brucei* in wild-type mice, there was substantial NO production in splenic and peritoneal

Table 3. T-cell proliferative response to concanavalin A in spleen cell cultures from *T. brucei*-infected and naive wild-type and IFN-γR$^{-/-}$ mice

Day of infection	Proliferative response (% of control)			
	Wild type	Wild type + L-NMMA	IFN-γ$^{-/-}$	IFN-γ$^{-/-}$ + L-NMMA
Day 3	99·6 ± 4·6	79·7 ± 3·9	129·3 ± 22·4	110·8 ± 14·5
Day 5	3·6 ± 1·6	23·2 ± 3·5	32·5 ± 13·2	35·6 ± 8·7
Day 7	1·2 ± 1·1	14·0 ± 4·6	39·2 ± 4·3	39·0 ± 9·3
Day 9	3·1 ± 1·0	6·7 ± 1·6	5·8 ± 2·3	5·7 ± 0·8

Data are expressed as mean percentage ± SD ($n=4$) of proliferation in uninfected controls at each time point (typically 80 000 counts per minute).

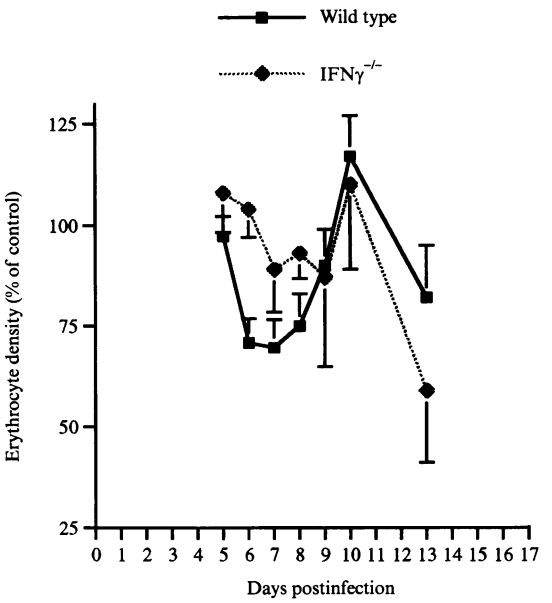

Figure 2. Onset of anaemia in wild-type and IFN-γR$^{-/-}$ mice. Data are expressed as mean percentage ± SE ($n=5$) of uninfected control erythrocyte concentration, typically 1×10^{10}/ml. These results are representative of two similar, independent experiments.

macrophages, while in IFN-γR$^{-/-}$ mice there was complete blockade of this response. Therefore, IFN-γR$^{-/-}$ mice provide an infection model in which the effects of NO are removed. Following *T. brucei*-infection of IFN-γR$^{-/-}$ mice, not only was parasitaemia increased but survival time was reduced over infected wild types. Similar results have also recently been reported by Bakhiet *et al.*[23]

Despite higher parasitaemia, both the suppression of T-cell proliferative responses and anaemia in the infected IFN-γR$^{-/-}$ mice were significantly reduced in comparison with infected wild-type mice during the first 10 days of infection. These results provide further evidence for the role of NO in immune suppression[7,8] and anaemia[9] in early murine trypanosome infection. Beyond day 10 of infection NO synthesis declined to preinfection levels, but both wild types and IFN-γR$^{-/-}$ mice displayed severe inhibition of T-cell proliferation and anaemia. This suggests that in chronic infection, suppressed T-cell proliferation and anaemia are mediated by NO-independent mechanisms, and is consistent with previous observations of NO-independent suppression in spleen[8] and lymph nodes[24] of chronically infected mice. The increased parasitaemia in *T. brucei*-infected IFN-γR$^{-/-}$ mice contrasts with previous studies using chemical inhibitors of NO synthesis, which caused reduction in parasitaemia during the first 10 days of infection.[12] This raises the question of why the IFN-γR$^{-/-}$ mice, which show no activation of iNOS during trypanosome infection, carry a higher parasitaemia than the wild types. It is likely that other activities of IFN-γ or activated macrophages, will explain these contradictory findings. For instance, it is possible that an increase in plasma IFN-γ, which has been predicted in trypanosome-infected IFN-γR$^{-/-}$ mice[23] and observed in such mice infected with bacillus Calmette–Guérin (BCG)[25] acts as a growth stimulus for *T. brucei*.[26] Also, it is possible that factors released by activated macrophages, other than NO, are involved in controlling *T. brucei* parasitaemia. In this context it is of interest that tumour necrosis factor-α (TNF-α) has been shown to have trypanocidal activity.[27]

In the light of the present study, it is possible to re-evaluate the relationship of macrophage activation, immunosuppression and anaemia in trypanosomiasis.[20,28] In acute murine infection, the primary mediators of suppression of T-cell proliferation are likely to be NO and other macrophage activation products such as PG. During this early period of infection, macrophage activation appears to be caused by a T helper 1 (Th1) cytokine response to trypanosome antigens,[14] although evidence has also been presented for NK cells[16] and CD8$^+$ T-cells[29] releasing IFN-γ in *T. brucei* infection. However, in chronic murine infection, T-cell unresponsiveness and anaemia are independent of NO, the synthesis of which is diminished. This might account for the lack of involvement of NO in immune suppression in bovine *T. conglolense* infection.[30] However, it is premature to exclude NO from consideration as a pathological mediator in human sleeping sickness where there is evidence of elevated NO synthesis in both acute and chronic infections.[31]

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