Clin Exp Immunol 1997; **110**:378–385

Trypanosoma cruzi induces strong IL-12 and IL-18 gene expression *in vivo*: correlation with interferon-gamma (IFN-γ) production

C. MEYER ZUM BÜSCHENFELDE, S. CRAMER, C. TRUMPFHELLER, B. FLEISCHER & S. FROSCH
Bernhard Nocht-Institute for Tropical Medicine, Department of Immunology, Hamburg, Germany

(Accepted for publication 2 September 1997)

SUMMARY

IFN-γ, produced after infection with *Trypanosoma cruzi*, has been shown to be crucial in the determination of resistance or susceptibility. We have performed a detailed study on the expression of IFN-γ and of the IFN-γ-inducing cytokines IL-12 and IFN-γ-inducing factor (IGIF)/IL-18 with regard to time course and tissue localization. IFN-γ was present in high amounts in the serum and in the supernatants of unseparated spleen cells and isolated $CD4^+$ and $CD8^+$ T cells from the spleens of infected mice which were stimulated *ex vivo* with *T. cruzi*. Using the *in situ* hybridization technique we demonstrate that IL-12 p40 messages were expressed in the spleen and increased during infection, correlating with the expression of IFN-γ transcripts. Furthermore, we show for the first time that the mRNA for the cytokine IL-18 was induced by a parasitic infection and that this expression increased during infection with *T. cruzi*. Interestingly, the message for IL-18 was produced earlier during infection and already had declined until day 38, when IFN-γ and IL-12 p40 transcripts were optimally expressed. Surprisingly, the changes in IL-12 and IL-18 mRNA production were clearly seen only by *in situ* hybridization, but less clearly by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). This is possibly due to the extensive activation and proliferation of spleen cells observed during infection leading to a dilution of these specific mRNAs.

Keywords *Trypanosoma cruzi* interferon-gamma IL-12 IL-18

INTRODUCTION

The protozoan homoflagellate *Trypanosoma cruzi* is the aetiologic agent of Chagas' disease in man and also infects a variety of mammalian species. Using various mouse strains, genetically susceptible or resistant to acute *T. cruzi* infection, it has been shown that T cell-mediated effector functions are essential for the control of the parasite load and host survival. Both $CD4^+$ [1–4] and $CD8^+$ [4–6] T cells contribute to the resistance against *T. cruzi*. One important mechanism by which T cells mediate the defence is through the secretion of lymphokines. Endogenous IFN-γ seems to play a central role in the control of infection [7,8]. Passive administration of IFN-γ results in an increased clearance of the parasite and a reduced mortality of infected mice [9], while anti-IFN-γ MoAbs augment the susceptibility to acute *T. cruzi* infection [10]. Furthermore, BALB.Xid mice that produce higher levels of IFN-γ than susceptible normal BALB/c mice are relatively resistant to infection [11]. The protective effect of IFN-γ involves the induction of nitric oxide (NO) synthase activity to produce NO, responsible for the microbicidal effect of macrophages for *T. cruzi* [12,13].

IFN-γ is produced by activated T cells and natural killer (NK) cells in the acute phase of infection [14]. A cytokine stimulating the production of IFN-γ from T cells and NK cells is IL-12, a 70-kD (p70) heterodimeric cytokine, composed of covalently linked 35-kD (p35) and 40-kD (p40) subunits. IL-12 strongly enhances the development and growth of Th1 cells, resulting in a protective immunity in mice infected with a variety of microorganisms [15,16]. Recently, it has been shown by Aliberti *et al*. [17] and by ourselves [18] that live trypanosomes induce high production of IL-12 p40 and biologically active IL-12 in spleen cells and bone marrow-derived macrophages *in vitro*. Furthermore, IL-12 seems to be responsible for the early IFN-γ production and for control of the parasite proliferation *in vivo* [17]. The protective effect of IL-12 in the regulation of parasitaemia depends on IFN-γ and tumour necrosis factor-alpha (TNF-α) [19].

Recently, a novel cytokine, called IFN-γ-inducing factor (IGIF), was purified from the livers of mice treated with *Propionibacterium acnes* and subsequently challenged with lipopolysaccharide (LPS) [20]. IGIF is readily detected in Kupffer cells and

Correspondence: Professor Dr Bernhard Fleischer, Bernhard Nocht-Institute for Tropical Medicine, Bernhard Nocht-Str. 74, D-20359 Hamburg, Germany.

activated macrophages. This cytokine possesses the capacity to induce IFN-γ production by spleen cells similarly to the structurally unrelated cytokine IL-12, but the mechanism by which IGIF induces IFN-γ seems to be different from that of IL-12. In addition, IGIF may be also involved in the development of Th1 cells and in the activation of NK cells [21]. Now, IGIF is called IL-18.

Here we have performed a detailed study of the kinetics of expression and the tissue distribution of IFN-γ and the IFN-γ-inducing cytokines IL-12 and IL-18 in mice after infection with *T. cruzi*. We demonstrate by *in situ* hybridization that after infection the messages for both IL-12 and IL-18 were induced and strongly increased during infection. Interestingly, the kinetic expression was somewhat different. Whereas IL-18 mRNA was already clearly visible at day 6 post-infection, IFN-γ and IL-12 p40 mRNAs were detectable at lower intensity and in less cells at this time. The expression of IFN-γ and IL-12 p40 increased steadily during infection, with maximal expression on days 26–38, whereas the expression of IL-18 transcripts had already declined at day 38. By quantifying the mRNA for these cytokines using the competitive reverse-transcriptase polymerase chain reaction (RT-PCR) technique, this up-regulation of IL-12 p40 and IL-18 transcripts was not clearly seen. This might be due to the strong proliferation and activation of spleen cells that leads to an increase in the total mRNA content, resulting in a strong dilution of the specific mRNAs tested.

MATERIALS AND METHODS

Mice and parasites

BALB/c mice, originally obtained from Charles River (Sulzfeld, Germany), were bred in our animal facilities. They were infected at 8 weeks of age. Two different strains of *T. cruzi*, the Tehuantepec strain [22] and the Tulahuén strain, were used. For simplicity and because similar results were obtained with both strains, only data for the Tehuantepec strain are presented. Trypomastigotes of the Tehuantepec strain were maintained in tissue culture by infection of 86HG39 glioblastoma cells [23]. Parasites leaving their host cells 10 days later were collected within the supernatant by centrifugation at 100 ***g*** to separate from cell debris and then centrifugation of the supernatant at 500 ***g***. The Tulahuén strain was maintained and propagated in BALB/c mice. Purification of the parasites from the peripheral blood was performed via a Ficoll-density gradient. Mice were infected by i.p. injection of 10^4 parasites in a volume of 0·2 ml PBS.

Spleen cell culture

A single-cell suspension of spleen cells, treated with Gey's solution for 1 min to lyse erythrocytes, was washed carefully and then resuspended in Iscove's modified Dulbecco's medium (IMDM), supplemented with 5×10^{-5} M 2-mercaptoethanol (2-ME), 50 μg/ml gentamycin, 2 mM L-glutamine, and 5% heat-inactivated fetal calf serum (FCS; complete IMDM). Cells (3×10^6/ml) were seeded in 24-well plates (Greiner, Nürtingen, Germany). Live trypomastigotes in a volume of 1 ml and in a cell:parasite ratio of 1:2 were added. As a negative control, cells were incubated with medium alone. On day 2 of culture, supernatants of the spleen cells were taken and assessed for IFN-γ content. All cultures were set up in triplicates. The standard error was < 10%. Results are representative of three experiments, each consisting of three animals, which were independently tested and gave similar results.

Isolation and activation of CD4$^+$ and CD8$^+$ T cells from the spleen

CD4$^+$ and CD8$^+$ T cells were isolated from spleen cells by magnetic cell separation according to the manufacturer's recommendation (Dynal, Hamburg, Germany). Cells were positive selected using rat MoAb GK1.5 (anti-CD4) [24] or 53.6.72 (anti-CD8) [25] and goat anti-rat IgG-labelled magnetic beads. CD4$^+$ and CD8$^+$ T cells (10^6) were stimulated with 4×10^6 irradiated (20 Gy) syngeneic spleen cells as antigen-presenting cells (APC) and 8×10^6 trypomastigotes in complete IMDM and were incubated in a total volume of 2 ml complete IMDM for 48 h. For control, T cells were stimulated with spleen cells in the absence of trypanosomes.

Isolation and infection of macrophages

Bone marrow-derived macrophages were generated and infected with trypanosomes and/or stimulated with recombinant rat IFN-γ (Life Technologies, Eggenstein, Germany) as previously described [18,26]. After 24 h macrophages were analysed for IL-12 p40 and IL-18 mRNA production.

IFN-γ assay

IFN-γ was detected by a two-site sandwich ELISA [27] using MoAb R4-6A2 and biotinylated AN18.17.24. A reference standard curve with murine rIFN-γ was included.

RNA isolation and cDNA synthesis

A single-cell suspension of spleen cells was treated with Tri-Reagent (Molecular Research Centre, Cincinnati, OH) and total RNA was prepared following the manufacturer's recommendation. Of the total RNA, 1 μg was reverse transcribed as described [18].

RT-PCR analysis

cDNA (4 μl; diluted 1:5) was used for PCR amplification in a 25 μl reaction, as described [18]. The primer pairs used for amplification were for *T. cruzi*: 5′-GACGGCAAGAACGCCAAGGAC-3′ and 5′-TCACGCGCTCTCCGGCACGTTGTC-3′; they amplify a 580-bp fragment. cDNA was also amplified for β-actin to control for the amount of input cDNA. Primers were: 5′-CTCTTTGATGTCACG-CACGATTTC-3′ and 5′-GTGGGCCGCTCTAGGCACCAA-3′. The product was a 535-bp fragment.

Quantitative RT-PCR using a multicompetitor molecule

The levels of β-actin, IFN-γ, IL-12 p40, and IL-18 transcripts were determined by competitive PCR using a self-constructed (unpublished data) multispecific control vector. After adjusting the cDNA samples to equal concentrations of β-actin, 3 μl of the diluted cDNA and specific primers were mixed with 3 μl of serial dilutions (two-to-five fold) of the competitor DNA and used for a PCR reaction, as described [18]. During the PCR reaction the control DNA and the cDNA compete for specific primers and are co-amplified, resulting in fragments that differ by about 90–100 bp in size. The PCR products derived from the control vector and the cDNA were resolved on a 1·5% agarose gel. By comparing their relative ethidium bromide staining intensities the relative amount of the respective cytokine cDNA in a given sample was determined. The sequences of the primers used were for β-actin: 5′-ATGGATGACGATATCGCT-3′ and 5′-ATGAGGTAGTCTGT-CAGGT-3′; for IFN-γ: 5′-AACGCTACACACTGCATCT-3′ and 5′-AGCTCATTGAATGCTTGG-3′; for IL-12 p40: 5′-ATGGC-CATGTGGGAGCTGGAG-3′ and 5′-TTTGGTGCTTCACACTT-CAGG-3′; for IGIF: 5′-ACTGTACAACCGCAGTAATACGG-3′

and 5′-AGTGAACATTACAGATTTATCCC-3′. The reaction was performed at the following temperatures: 3 min 95°C, 30 s 95°C, 30 s 68°C/63°C/58°C (each five cycles), 90 s 72°C, 30 s 95°C, 30 s 55°C (12 cycles for *β*-actin, 20 cycles for IFN-γ, 25 cycles for IGIF, and 30 cycles for IL-12 p40), and 90 s 72°C.

Preparation of ^{35}S-labelled RNA probes and in situ *hybridization*

The IFN-γ and the IL-12 p40 probes have been described [28,29]. IGIF- [21] and *T. cruzi*- [30] specific probes were cloned in the Bluescript KS vector (Stratagene, Heidelberg, Germany). Subcloning of specific DNA fragments in plasmids with SP6 or T3/T7 initiation sites (Boehringer, Mannheim, Germany) was done by standard protocols. *In vitro* transcription of sense and antisense probes was performed as described [28,29].

In situ hybridization using ^{35}S-labelled *in vitro* transcribed cRNA probes on cryostat section was performed as described previously [28,29]. After 15 days at 4°C sections were developed in Kodak D-19 developer and counterstained with haemalaun. As negative controls, sections for each time point were hybridized with ^{35}S-labelled sense-strand probes of each cytokine. For simplicity only the autoradiogram for the IL-12 p40 sense probe of one time point is shown.

RESULTS

Expression of T. cruzi*-specific mRNA in the spleen after infection of mice*

Two strains of *T. cruzi* were used to analyse expression of IFN-γ and the IFN-γ-inducing cytokines IL-12 and IGIF in mice. While mice infected with the strain Tulahuén died 17–21 days after i.p. infection, mice inoculated with the strain Tehuantepec survived. Live trypomastigotes of both strains appeared in the lymphatic organs like the spleen and mesenterial lymph nodes already 3 days after infection. Because we were interested to follow up the immune response induced by *T. cruzi* for a longer period of time and because we observed similar cytokine production in the early period after infection with both strains, only the data obtained with the strain Tehuantepec are presented here. It is noteworthy that spleens of infected mice showed a marked enlargement, which was due to an intense proliferation of all major cellular subpopulations, most notably $CD4^+$ and $CD8^+$ T cells, B cells, and to a lesser degree macrophages. The cells appeared as big blasts. The total number of spleen cells increased about five times (data not shown).

As shown by RT-PCR using a *T. cruzi*-specific primer pair (Fig. 1a), trypanosoma-specific mRNA was detectable from day 3 on in the spleen. Specific mRNA expression peaked on day 26 and

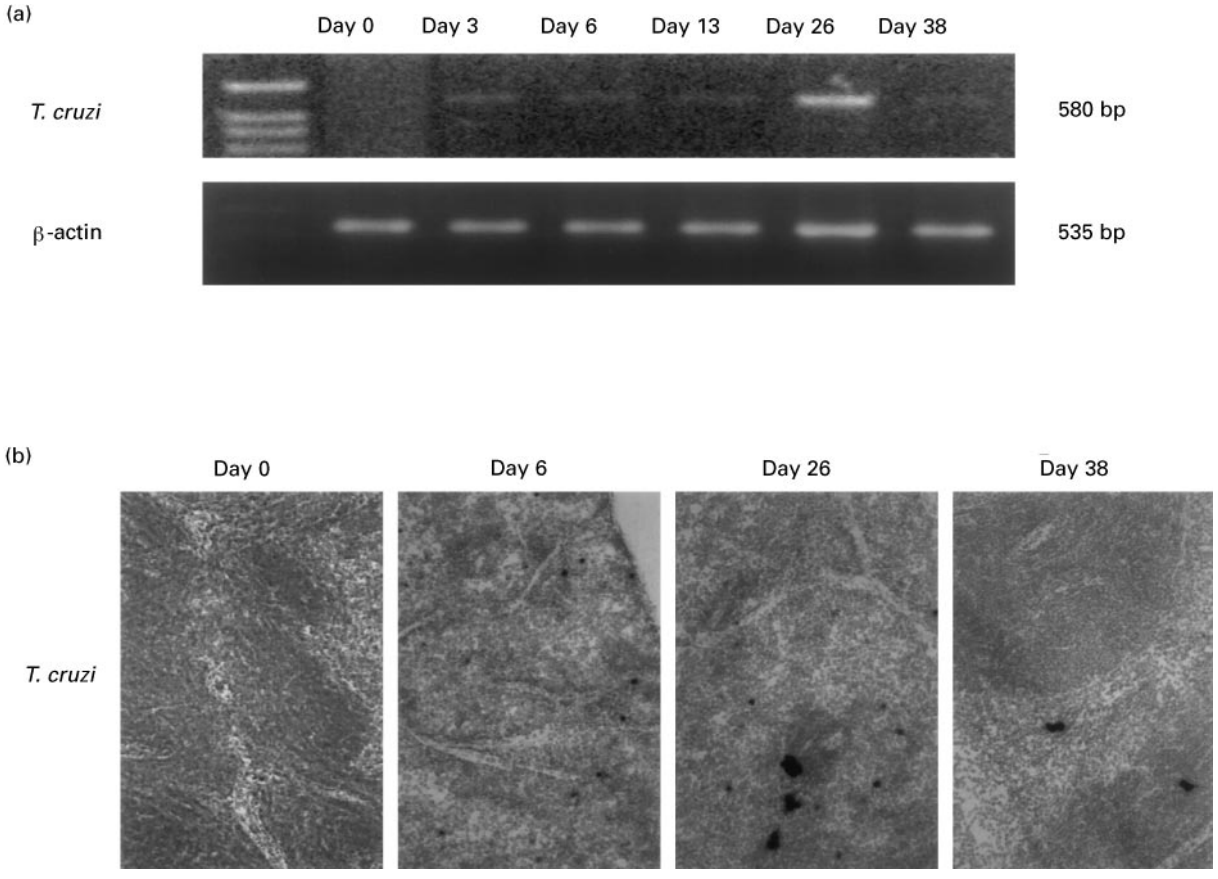

Fig. 1. Expression of *Trypanosoma cruzi* mRNA in the spleens of infected mice. (a) At different time points after infection with *T. cruzi*, RNA from the spleens was prepared and reverse transcribed. From each sample cDNA was subjected to reverse-transcriptase polymerase chain reaction (RT-PCR) for detection of *T. cruzi* mRNA and *β*-actin mRNA for control. (b) Sections of spleens obtained at different time points were hybridized with a ^{35}S-labelled antisense RNA probe for *T. cruzi*. Results are representative of two experiments using five mice in each experiment.

declined thereafter. Similar results were obtained using the method of *in situ* hybridization. For simplicity, only a few time points are presented (Fig. 1b). On day 6, infected cells were detectable in the spleen. However, the intensity of the hybridization signals was low. During infection till day 26, however, there was a strong increase of the hybridization signal, although the number of infected cells did not rise. The trypanosomes were distributed over the whole spleen. No specific enrichment to any particular microenvironment was obvious. Interestingly, on day 38 fewer cells were positive for *T. cruzi* mRNA. Thus, it seems that the mice were able to eliminate most of the trypanosomes in the tissue. The decline in trypanosomes in the tissue correlated with the enhancement of the expression of mRNA for the enzyme-inducible NO synthase (iNOS), responsible for the generation of NO (data not shown). NO has microbicidal function for trypanosomes [12,13].

Trypanosoma cruzi *is a potent inducer of IFN-γ production* in vivo

IFN-γ is known to play a central role in the control of infection with *T. cruzi* [7–10]. In accordance with previous reports using other *T. cruzi* strains, we found using the Tehuantepec and Tulahuén strains that IFN-γ is the predominant cytokine produced after infection of BALB/c mice. IFN-γ was even detectable in the sera of mice infected with *T. cruzi* from day 6 on, peaked on day 13 of infection and declined thereafter (Fig. 2a). Neither IL-4 nor IL-10 were detected at any time in the serum (data not shown). Furthermore, IFN-γ was also produced by spleen cells from infected mice (Fig. 2b). After *ex vivo* stimulation with trypanosomes, spleen cells of infected mice were induced to produce IFN-γ, with peak production at 13 days post-infection. In the absence of trypanosomes at all time points IFN-γ was not produced.

Trypanosoma cruzi *induces IFN-γ production in CD4$^+$ and CD8$^+$ T cells isolated from the spleens of infected mice*

To investigate which T cell subpopulation was capable of producing IFN-γ, spleen cells from infected mice were separated into CD4$^+$ and CD8$^+$ T cells and stimulated *ex vivo* with live trypanosomes in the presence of syngeneic spleen cells as APC. Both CD4$^+$ T cells and CD8$^+$ T cells (Table 1), stimulated with trypanosomes, contributed to the high IFN-γ production seen in unseparated spleen cells. Interestingly, CD4$^+$ T cells produced higher amounts of IFN-γ than CD8$^+$ T cells. Also here, maximal IFN-γ production was seen when T cells were isolated from the spleens of mice infected for 13 days. Also, CD4$^-$CD8$^-$ cells strongly secreted IFN-γ after stimulation with *T. cruzi* (data not shown).

Detection of IL-12 p40- and IL-18-producing cells in the spleens of infected mice

Because of the large amounts of IFN-γ produced during infection with *T. cruzi*, we investigated whether infection of mice with *T. cruzi* results in the induction of the expression *in vivo* of IL-12 and IL-18, which are both potent inducers of IFN-γ production [15,16,21]. To localize individual cells producing the cytokines IFN-γ, IL-12 and IL-18 in the spleen, we performed *in situ* hybridization experiments. As shown in Fig. 3, hybridization signals were absent in the spleens of uninfected mice. On day 6 after infection only very few cells in the spleen showed positive IFN-γ-specific hybridization signals. Interestingly, the number of infected cells did not increase during infection, but the intensity of the signals drastically increased. In parallel to the enhancement

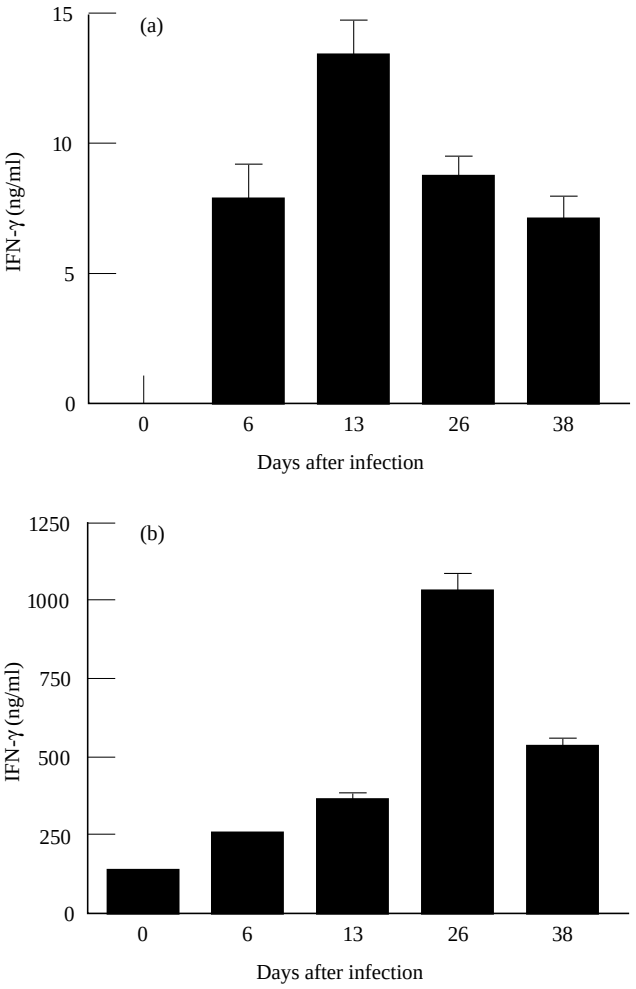

Fig. 2. IFN-γ production in *Trypanosoma cruzi*-infected mice. (a) Serum of mice infected with *T. cruzi* was taken at different time points after infection and assessed for IFN-γ content by a two-sided sandwich ELISA. (b) Spleen cells (4×10^6) of infected and non-infected mice were cultivated in medium alone or were *ex vivo* stimulated with live *T. cruzi*. After 48 h, supernatants were assessed for IFN-γ content by a specific ELISA. Results are representative of three experiments, each consisting of three animals, which were independently tested.

of IFN-γ signals, mRNA expression of IL-12 p40 in the spleen increased steadily during infection, with maximal expression on days 26–38. In contrast to IFN-γ expression, however, both the intensity of the signals and the number of IL-12 p40 mRNA-expressing cells increased. Clearly, more cells expressing IL-18 mRNA were already seen in the early stage of infection (day 6). On day 26 the number of positive cells and the strength of the signal were very high. Both the intensity of the signal and the number of cells expressing IL-18 mRNA declined when the infection proceeded, in contrast to the high abundance of cells expressing either IL-12 p40 or IFN-γ mRNAs until day 38 post-infection. Thus, it is remarkable that IL-18 and IL-12 p40 mRNAs showed somewhat distinct kinetics. IL-18 mRNA increased earlier during infection than IL-12 p40 mRNA. Most of the cells producing IFN-γ, IL-12 p40, and IL-18 mRNAs were found in the white pulp, especially in the periarterial lymphatic sheath (PALS), while only a small percentage of cells was detectable in the red pulp. Cells with positive hybridization signals for IFN-γ, IL-12 p40 and

Table 1. Production of IFN-γ by $CD4^+$ and $CD8^+$ T cells from infected mice after *ex vivo* stimulation

	IFN-γ (ng/ml) in the supernatants of			
	$CD4^+$ T cells + spleen cells		$CD8^+$ T cells + spleen cells	
Time after infection	Without *T. cruzi*	With *T. cruzi*	Without *T. cruzi*	With *T. cruzi*
Day 0	0	0	0	0
Day 6	0	13·5	0	0
Day 13	0	23·6	0	5·9
Day 26	0	3·5	0	3·1
Day 38	0	2·4	0	4·9

Mice were infected with *Trypanosoma cruzi* (10^4 trypomastigotes) for different times or injected with PBS for control (day 0). Splenic $CD4^+$ or $CD8^+$ T cells were isolated by magnetic cell separation and stimulated (10^6/well) *ex vivo* with irradiated (20 Gy) syngeneic spleen cells (4×10^6/well) in the presence or absence of *T. cruzi* (8×10^6/well) as antigen in a total volume of 2 ml. After 48 h supernatants were taken and assessed for IFN-γ.

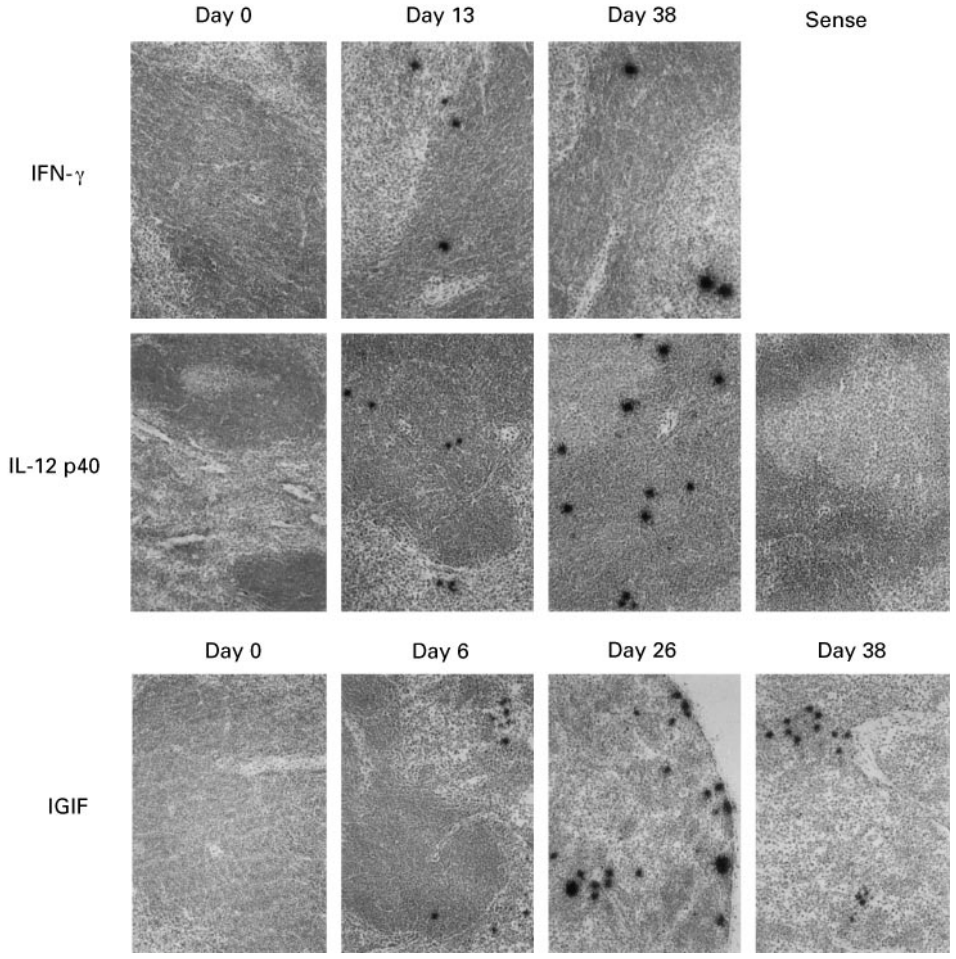

Fig. 3. Kinetics and distribution of *Trypanosoma cruzi*-induced IFN-γ, IL-12 p40, and IL-18 mRNAs in the spleen. Serial sections of spleens, taken at different time points after infection, were hybridized with ^{35}S-labelled cRNA probes for IFN-γ, IL-12 p40, and IL-18 (IGIF) and evaluated by autoradiography. Results are representative of two experiments.

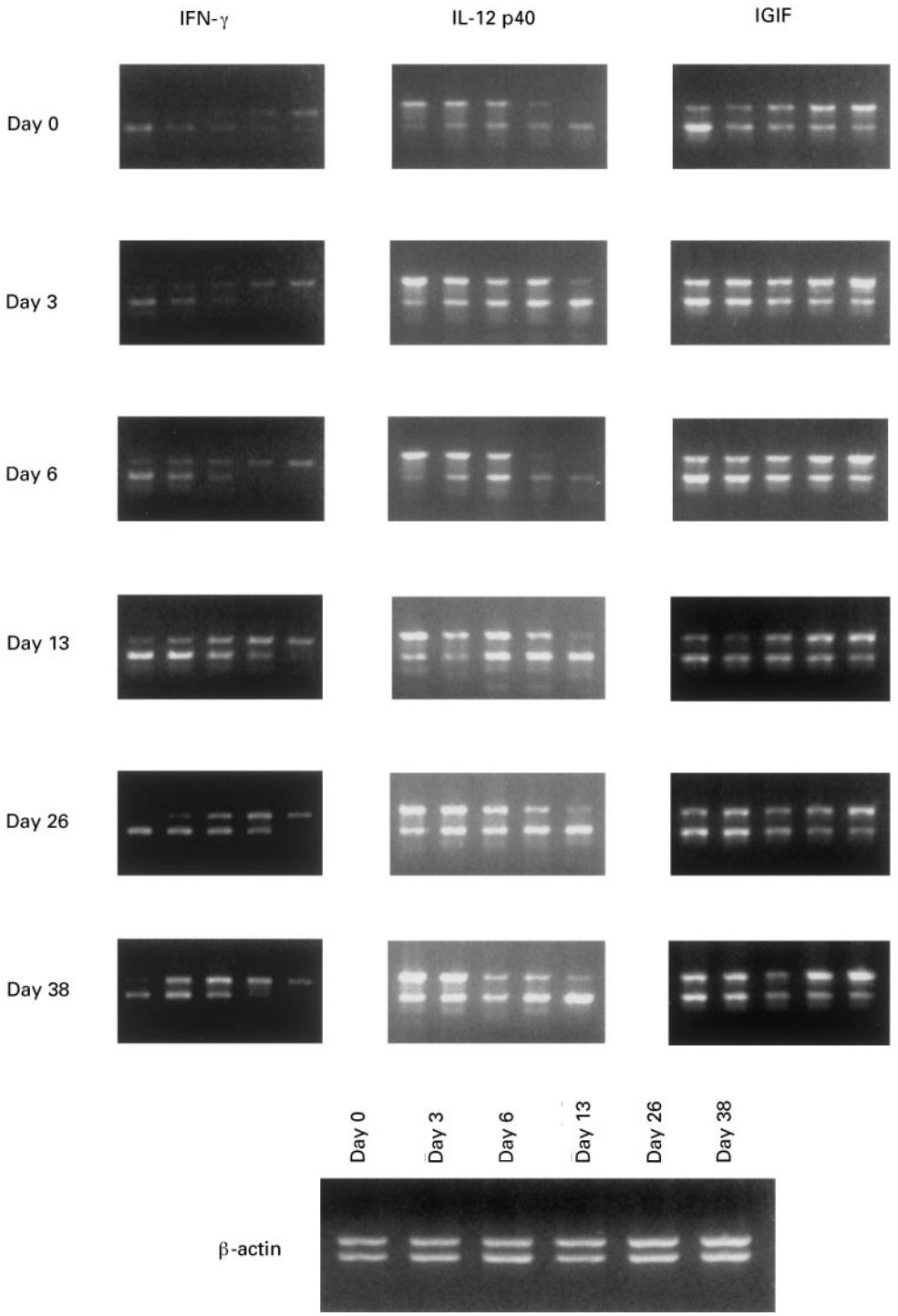

Fig. 4. Quantification of IFN-γ, IL-12 p40, and IL-18 mRNAs. Mice were infected with *Trypanosoma cruzi* (10^4) for different time points. Spleens were harvested at time 0 (control animals) and 3, 6, 13, 26 and 38 days post-infection and frozen in Tri-Reagent. mRNA preparations were reverse transcribed and after adjusting for equal amounts of cDNA for *β*-actin the messages for IFN-γ, IL-12 p40 and IL-18 (IGIF) were amplified by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) in the presence of a polycompetitor molecule. Primers for IFN-γ amplify a 569-bp fragment of the cDNA and a 483-bp fragment of the competitor, primers for IL-12 p40 amplify a 335-bp fragment of the cDNA and a 434-bp fragment of the competitor, primers for IGIF amplify a 434-bp fragment of the cDNA and a 333-bp fragment of the competitor. For quantification of IFN-γ the control vector was serially five-fold diluted, starting at a dilution of 1:3125 (days 0, 3 and 6) or 1:125 (days 13, 26 and 38). For quantification of IL-12 p40 the control vector was serially two-fold diluted, starting at a dilution of 1:200 000. For quantification of IL-18 serial two-fold dilutions were performed, starting at a dilution of 1:6250. All PCR results shown were independently repeated two-to-three times to confirm results.

Table 2. Expression of IL-18-specific mRNA in macrophages after infection with *Trypanosoma cruzi*

Stimulation of macrophages* with	Relative mRNA content† of	
	IL-12 p40	IL-18
–	0·066	6·4
T. cruzi	8·192	12·8
T. cruzi + IFN-γ	8·192	25·6

*Aliquots of bone marrow-derived macrophages were infected for 24 h with *T. cruzi* at a parasite:cell ratio of 10:1, were infected with trypanosomes in the presence of IFN-γ (10 U/ml), or were left untreated.

†Total RNA was isolated from the macrophages and reverse transcribed. cDNA from each sample was subjected to quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for detection of IL-12 p40 and IL-18 mRNA. Values represent the ratio of target gene:β-actin gene expression ($\times 10^{-3}$).

IL-18 represented single cells and did not build clusters in the spleen.

To quantify IFN-γ, IL-12 p40, and IL-18 mRNA production in the spleens of infected mice we performed quantitative RT-PCR studies using a multicompetitor molecule. As shown in Fig. 4, the message for IFN-γ increased 25 times to day 13 after infection, and was further enhanced five times to day 38. Surprisingly, a clear up-regulation after infection of the messages for IL-12 p40 and IL-18 was not detectable by this technique.

IL-18-specific transcripts are induced in vitro *after infection of macrophages with* T. cruzi

Since macrophages are the major host cells during infection with *T. cruzi*, we extended the *in vivo* studies and infected bone marrow-derived macrophages with *T. cruzi in vitro* and performed quantitative RT-PCR studies. As shown in Table 2, uninfected macrophages already expressed IL-18 messages. Infection with *T. cruzi* only slightly enhanced IL-18 mRNA expression. In the presence of IFN-γ trypanosomes enhanced IL-18 transcription in the macrophages about four times. This up-regulation was much lower than the up-regulation of IL-12 p40 transcripts. Furthermore, IL-12 p40 mRNA was already induced in high amounts by the trypanosomes alone in the absence of IFN-γ, as shown previously [18].

DISCUSSION

IFN-γ plays a critical role in resistance to infection with *T. cruzi* [7–10]. Here, we have investigated the distribution and kinetics of the gene expression of IFN-γ and of two cytokines that stimulate its production. When both the Tehuantepec strain (data presented here) and the Tulahuén strain (data not shown) of *T. cruzi* were used, the predominant cytokine induced by the parasite *in vivo* was IFN-γ. The message for IFN-γ increased about 125 times from day 0 to day 38 after infection. Also, in a kinetic analysis we could demonstrate that maximal levels of IFN-γ were obtained in the serum and spleen between 13 and 26 days after infection. Both $CD4^+$ and, to a lesser degree, $CD8^+$ T cells contributed to this strong production, as shown by *ex vivo* stimulation assays. Furthermore, $CD4^-CD8^-$ cells secreted high amounts of IFN-γ (data not shown), and NK cells were also described to produce IFN-γ after *in vitro* stimulation with trypanosomes [14].

It is well established that IL-12 not only enhances the cytolytic activity and proliferation of T cells but also induces IFN-γ synthesis [15,16]. Consistent with these findings, we and others have recently shown that IL-12 p40 mRNA and biologically active IL-12 are produced by spleen cells and macrophages infected *in vitro* with trypanosomes [17,18]. Moreover, we demonstrate here that infection with *T. cruzi* also triggers IL-12 mRNA synthesis *in vivo*. As shown by *in situ* hybridization, a sensitive assay for cytokine production in individual cells, the number of cells expressing IL-12 messages and the strength of the signal increased during infection. *In situ* hybridization shows the producing cells at a particular moment. It is noteworthy that the IL-12 signal is extremely strong, nearly equivalent to the amount of IL-12 produced after polyclonal *in vivo* stimulation with the superantigen Staphylococcal enterotoxin B [29]. In parallel experiments, we could detect only very weak signals for IL-12 in B6 mice infected with *Leishmania major* (unpublished observations), although these mice mount an IL-12-dependent Th1 response to this parasite. The importance of our finding is underlined by the recent report that *in vivo* neutralization of IL-12 by antibodies results in a significantly increased parasitaemia and mortality of infected mice [17]. The important role of IL-12 in controlling parasitaemia in *T. cruzi*-infected mice was further confirmed by Hunter *et al*. [19], who demonstrated that mice treated with IL-12 during infection with trypanosomes have a reduced parasitaemia. Furthermore, a significantly enhanced survival time compared with infected but untreated controls was observed.

Recently the IFN-γ-inducing factor IGIF/IL-18 was identified and cloned [20], that similarly to IL-12 stimulates T cell proliferation, augments the lytic activity of cells and, most importantly in this context, costimulates IFN-γ production in splenic T cells and in cloned Th1 cells in the presence of anti-CD3 MoAb. Interestingly, the action of IL-18 is independent of the presence of IL-12, as shown by IL-12-specific antibodies. Moreover, anti-IGIF MoAbs do not inhibit IFN-γ induction by IL-12, confirming that these cytokines act independently of each other [21]. *In vivo*, IL-18 was detected in the sera of mice treated with *P. acnes* and challenged with LPS. Here we have demonstrated for the first time the production of IL-18 in a parasitic infection. Not only did a pure macrophage population infected *in vitro* with trypanosomes express IL-18 transcripts in the presence of IFN-γ, but also *in vivo* we were able to detect IL-18 mRNA in the spleens of mice infected with *T. cruzi* using *in situ* hybridization techniques. Early after infection, IL-18 mRNA-positive cells were present in the spleen. Not only the number of cells producing IGIF mRNA but also the strength of the hybridization signal increased. Both IL-12 and IL-18 mRNA were localized in the T cell areas. It was unexpected that the enhancement of IL-12 and IL-18 mRNA expression detected by *in situ* hybridization was not clearly seen in quantitative RT-PCR studies. A possible explanation is the extremely strong activation and proliferation of all cellular subpopulations observed. Due to a high cellular metabolism the total amount of the mRNA increased, resulting in a strong dilution of the IL-12 p40 and IL-18 transcripts. Thus, for detection of inducible mRNAs by infectious agents which cause a strong cellular activation it is not sufficient to perform only RT-PCR studies. A combination of quantitative RT-PCR and *in situ* hybridization studies might be useful and necessary.

Because of its potential to induce IFN-γ production, it is most likely that IL-18 contributes to the strong IFN-γ expression of mice

seen after *T. cruzi* infection. Although it would be necessary to perform neutralization studies, inhibiting IL-18 *in vivo*, to confirm this hypothesis, a comparison of the kinetics of appearance of IL-18 and IL-12 p40 messages in the tissue shows that IGIF transcripts appeared somewhat earlier during infection, while transcription of IL-12 on the other hand proceeded for a longer time period. Therefore, one could speculate that expression of the IL-18 gene is of particular importance for induction of IFN-γ production early after infection, while later on IL-12 is responsible for supporting and maintaining IFN-γ production.

In their response to many microbes, macrophages produce IL-12 which stimulates the development of the T cell subset, whose function is to enhance the microbicidal activity of the macrophages. In many intracellular infections, e.g. listeriosis [31] and toxoplasmosis [32], IL-12 is induced early during infection and plays a central role in establishing IFN-γ-dependent resistance as well as polarized Th1 response profiles. Our data suggest that IL-18 may also turn out to be an important regulator protein for induction of an appropriate type of immune defence against intracellular parasites.

ACKNOWLEDGMENTS

This work was supported by the Fonds der Chemischen Industrie and by the Volkswagen-Stiftung. We thank A. Veit and S. Kraus for expert technical assistance.

REFERENCES

1 Russo M, Starobinas N, Minoprio P, Coutinho A, Hontebeyrie Joskowicz M. Parasitic load increases and myocardial inflammation decreases in *Trypanosoma cruzi*-infected mice after inactivation of helper T cells. Ann Inst Pasteur Immunol 1988; **139**:225–36.

2 Araujo F. Development of resistance to *Trypanosoma cruzi* in mice depends on a viable population of L3T4$^+$ (CD4$^+$) T lymphocytes. Infect Immun 1989; **57**:2246–8.

3 Rottenberg M, Cardoni RL, Andersson R, Segura EL, Orn A. Role of T helper/inducer cells as well as natural killer cells in resistance to *Trypanosoma cruzi* infection. Scand J Immunol 1988; **28**:573–82.

4 Rottenberg ME, Bakhiet M, Olsson T *et al*. Differential susceptibilities of mice genomically deleted of CD4 and CD8 to infections with *Trypanosoma cruzi* or *Trypanosoma brucei*. Infect Immun 1993; **61**:5129–33.

5 Tarleton RL. Depletion of CD8$^+$ T cells increases susceptibility and reverses vaccine-induced immunity in mice infected with *Trypanosoma cruzi*. J Immunol 1990; **144**:717–24.

6 Tarleton RL, Koller BH, Latour A, Postan M. Susceptibility of β_2-microglobulin-deficient mice to *Trypanosoma cruzi* infection. Nature 1992; **356**:338–40.

7 Torrico F, Heremans H, Rivera MT, Van Marck E, Billiau A, Carlier Y. Endogenous IFN-γ is required for resistance to acute *Trypanosoma cruzi* infection in mice. J Immunol 1991; **146**:3626–32.

8 Silva JS, Morrissey PJ, Grabstein KH, Mohler KM, Anderson D, Reed SG. Interleukin 10 and interferon γ regulation of experimental *Trypanosoma cruzi* infection. J Exp Med 1992; **175**:169–74.

9 Reed SG. *In vivo* administration of recombinant IFN-γ induces macrophage activation, and prevents acute disease, immune suppression, and death in experimental *Trypanosoma cruzi* infection. J Immunol 1988; **140**:4342–7.

10 Petray PB, Rottenberg ME, Bertot G *et al*. Effect of anti-γ-interferon and anti-interleukin-4 administration on the resistance of mice against infection with reticulotropic and myotropic strains of *Trypanosoma cruzi*. Immunol Letters 1993; **35**:77–80.

11 Minoprio P, El Cheikh MC, Murphy E *et al*. Xid-associated resistance to experimental Chagas' disease is IFN-γ dependent. J Immunol 1993; **151**:4200–8.

12 Gazzinelli RT, Oswald IP, Hieny S, James SL, Sher A. The microbicidal activity of interferon-γ-treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by interleukin-10 and transforming growth factor-β. Eur J Immunol 1992; **22**:2501–6.

13 Munoz-Fernandez MA, Fernandez MA, Fresno M. Synergism between tumor necrosis factor-α and interferon-γ on macrophage activation for the killing of intracellular *Trypanosoma cruzi* through a nitric oxide-dependent mechanism. Eur J Immunol 1996; **22**:301–7.

14 Cardillo F, Voltarelli JC, Reed SG, Silva JS. Regulation of *Trypanosoma cruzi* infection in mice by γ interferon and interleukin 10: role of NK cells. Infect Immun 1996; **64**:128–34.

15 Trinchieri G. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. Blood 1994; **84**:4008–27.

16 Wolf SF, Sieburth D, Sypek J. Interleukin 12: a key modulator of immune function. Stem Cells 1994; **12**:154–68.

17 Aliberti JC, Cardoso MA, Martins GA, Gazzinelli RT, Vieira LQ, Silva JS. Interleukin-12 mediates resistance to *Trypanosoma cruzi* in mice and is produced by murine macrophages in response to live trypomastigotes. Infect Immun 1996; **64**:1961–7.

18 Frosch S, Kraus S, Fleischer B. *Trypanosoma cruzi* is a potent inducer of interleukin-12 production in macrophages. Med Microbiol Immunol 1996; **185**:189–93.

19 Hunter CA, Slifer T, Araujo F. Interleukin-12-mediated resistance to *Trypanosoma cruzi* is dependent on tumor necrosis factor alpha and γ interferon. Infect Immun 1996; **64**:2381–6.

20 Nakamura K, Okamura H, Nagata K, Komatsu T, Tamura T. Purification of a factor which provides a costimulatory signal for γ interferon production. Infect Immun 1993; **61**:64–70.

21 Okamura H, Tsutsui H, Komatsu T *et al*. Cloning of a new cytokine that induces IFN-γ production by T cells. Nature 1995; **378**:88–91.

22 Rivera-Vanderpas MT, Rodriguez AM, Afchain D, Bazin H, Capron A. *Trypanosoma cruzi*: variation in susceptibility of inbred strains of rats. Acta Trop 1983; **40**:5–10.

23 Bilzer T, Stavrou D, Dahme E *et al*. Morphological, immunocytochemical and growth characteristics of three human glioblastomas established *in vitro*. Virchows Arch A Pathol Anat Histopathol 1991; **418**:281–93.

24 Wilde DB, Marrack P, Kappler J, Dialynas DP, Fitch FW. Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK1·5 (anti-L3T4a) blocks class II MHC antigen-specific proliferation, release of lymphokines, and binding by cloned murine helper T lymphocyte lines. J Immunol 1983; **131**:2178–83.

25 Ledbetter JA, Herzenberg LA. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol Rev 1979; **47**:63.

26 Frosch S, Küntzlin D, Fleischer B. Infection with *Trypanosoma cruzi* selectively upregulates B7–2 molecules on macrophages and enhances their costimulatory activity. Infect Immun 1997; **65**:971–7.

27 Hsieh C-S, Heimberger AB, Gold JS, O'Garra A, Murphy KM. Differential regulation of T helper phenotype development by interleukins-4 and -10 in an Ab -T cell-receptor transgenic system. Proc Natl Acad Sci USA 1992; **89**:6065–9.

28 Bette M, Schäfer MK, van Rooijen N, Weihe E, Fleischer B. Distribution and kinetics of superantigen-induced cytokine gene expression in mouse spleen. J Exp Med 1993; **178**:1531–9.

29 Bette M, Jin SC, Germann T *et al*. Differential expression of mRNA encoding interleukin-12 p35 and p40 subunits *in situ*. Eur J Immunol 1994; **24**:2435–40.

30 Ouaissi A, Aguirre T, Plumas-Marty B *et al*. Cloning and sequencing of a 24-kDa *Trypanosoma cruzi* specific antigen released in association with membrane vesicles and defined by a monoclonal antibody. Biol Cell 1992; **75**:11–17.

31 Tripp CS, Gately MK, Hakimi J, Ling P, Unanue ER. Neutralization of IL-12 decreases resistance to Listeria in SCID and C.B-17 mice. Reversal by IFN-γ. J Immunol 1994; **152**:1883–7.

32 Gazzinelli RT, Wysocka M, Hayashi S *et al*. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. J Immunol 1994; **153**:2533–43.