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Tumor necrosis factor-α in combination with interferon-γ, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes*

We have previously shown that during an infection with *Leishmania major*, susceptible BALB/c mice, as opposed to mice of a resistant strain (C57BL/6), are primed by lipopolysaccharide for the production of high levels of tumor necrosis factor-α (TNF-α) which is known to be a potent macrophage (MΦ) stimulator in other parasitic diseases. In the present study we investigated whether TNF-α activates MΦ for killling of *L. major* parasites. In the absence of interferon-γ (IFN-γ) or lipopolysaccharide, TNF-α (0.025-25000 U/ml) failed to activate peritoneal exudate MΦ from BALB/c mice for killling of *L. major* amastigotes. In the presence of suboptimal doses of IFN-γ (5 or 10 U/ml), however, TNF-α mediated a rapid elimination of intracellular parasites, which was highly significant compared to IFN-γ alone. The combination of TNF with interleukin 4, in contrast, was inactive in this respect and allowed survival of intracellular parasites. From these data we conclude that the presence of IFN-γ is crucial for TNF-α-mediated killing of *L. major* parasites by MΦ. Disease progression in susceptible mice therefore seems to be a consequence of a deficiency of IFN-γ and a predominance of interleukin 4 rather than the result of an excess amount of TNF-α.

1 Introduction

Leishmania are protozoan parasites which, in their amastigote form (AM), multiply within the phagolysosomes of non-activated MΦ [1]. The intracellular survival of AM is prevented when the MΦ are stimulated by T cell-derived lymphokines [2]. It has been shown that IFN-γ is the most important component among these MΦ-activating mediators [3, 4], although other cytokines have also been implicated in anti-parasitic effects [5-9]. The role of IFN-γ as the predominant lymphokine responsible for the ultimate elimination of Leishmania from MΦ is further illustrated by the fact that a *Leishmania major* infection in resistant C57BL/6 healer mice is associated with the expansion of IFN-γ-producing $CD4^+$ T lymphocytes, whereas in susceptible BALB/c mice, which finally succumb to the disease, IL 4-secreting $CD4^+$ T lymphocytes prevail [10].

Recently, we have demonstrated that mice infected with *L. major* are primed for the production of TNF-α *in vivo* (H. Moll, K. Binöder, C. Bogdan, W. Solbach and M. Röllinghoff, *Parasite Immunol.*, in press). We also found that during the course of an *L. major* infection, susceptible BALB/c mice elicit a significantly higher LPS-induced TNF-α response than resistant C57BL/6 mice. In other experimental systems the production of TNF-α has been associated with either protection [11-14] or damage of the host organism [15-17]. These results together with a number of reports on the capacity of TNF-α to activate MΦ for parasite elimination [18-21] prompted us to evaluate the effect of TNF-α on *L. major*-infected MΦ *in vitro*. In the studies to be presented we demonstrate that TNF-α alone failed to activate murine MΦ for killing of *L. major* amastigotes. However, in the presence of very low, suboptimal dosages of IFN-γ (5 or 10 U/ml) TNF-α induced rapid parasite degradation by the MΦ. In contrast, TNF-α in combination with IL 4 supported intracellular parasite survival. The implications of these findings for the pathogenesis of murine *L. major* infections will be discussed.

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Abbreviations: **L. major:** *Leishmania major* **AM:** *L. major* amastigotes

2 Materials and methods

2.1 Mice and parasite

Female BALB/c mice, weighing 16-18 g, were purchased from Charles River Breeding Laboratories (Sulsfeld, FRG) and were used at the age of 6-8 weeks. The origin and propagation of the *L. major* strain used as well as the *in vitro* culture of promastigotes was reported in detail previously [22]. AM suspensions were prepared from skin lesions of BALB/c mice 2-3 weeks after inoculation of 2×10^7 promastigotes as described [23]. Briefly, infected tissue was disrupted and homogenized by passage through a stainless steel mesh into culture medium. AM were released from MΦ by two vigorous passages of the primary cell suspension through a 27-gauge needle. Cell debris was removed by two steps of centrifugation at $120 \times g$ (10 min), before the AM were recovered ($1800 \times g$, 10 min) and washed twice.

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2.2 Preparation of MΦ cultures

T cell-free MΦ monolayers were prepared and tested for purity as described in detail elsewhere [23]. Briefly, thioglycolate-induced peritoneal exudate cells were washed twice, resuspended in C-RPMI culture medium 1640 (Biochrom, Berlin, FRG; supplemented with 2 mM L-glutamine, 10 mM Hepes buffer, 7.5% $NaHCO_3$, 0.05 mM 2-ME, 100 µg/ml penicillin, 160 µg/ml gentamycin and 10% selected FCS with a total LPS content <0.6 ng/ml), seeded into Labtek tissue culture chambers (2×10^5/chamber; Flow Laboratories, Meckenheim, FRG) and were allowed to adhere for 3–4 h (at 37 °C, 5% CO_2, 95% air humidity); thereafter, nonadherent cells were removed by three extensive washings with culture medium. Further incubation was performed either in culture medium or in culture medium with various cytokines as indicated in the text.

2.3 Cytokines and LPS

Murine rTNF-α [sp. act. 2.5×10^7 U/mg as determined by the supplier in a bioassay with murine tumor cells; LPS content of <0.025 ng/ml as determined in the limulus amebocyte lysate (LAL) assay] and murine rIFN-γ [sp. act. 1×10^7–2×10^7 U/mg as determined by the supplier in a bioassay with mouse L cells; LPS content (LAL assay) 0.0125 ng/ml] were kindly provided by Dr. G. R. Adolf (Ernst-Boehringer-Institut, Vienna, Austria). Murine rIL 4 [biological activity: 1.4×10^5 U/ml, 1 U/ml gives half maximum stimulation of cells of the HT-2 cell line; LPS contents (LAL assay): 0.5–5 ng/ml] was a gift from Dr. R. Coffman (DNAX Research Institute, Palo Alto, CA]. LPS (*E. coli* serotype 055:B5) was purchased from Difco (Detroit, MI).

2.4 MΦ stimulation with cytokines, infection of MΦ and assessment of intracellular parasites

Prior to infection, MΦ monolayers were incubated overnight either in culture medium alone or in culture medium with LPS or cytokine(s). The MΦ were then infected with AM (parasite : cell ratio = 5 : 1) for 3 h in the respective medium. Thereafter, non-phagocytosed parasites were washed off, and the cultures were further incubated in the respective medium, which was replaced every 24 h. Intracellular live amastigotes were assessed as described in detail elsewhere [24]. Briefly, infected MΦ monolayers were incubated with a solution of acridine orange (5 µg/ml) and ethidium bromide (50 µg/ml) in PBS for 10 min. After two washings the stained monolayers were fixed with paraformaldehyde (1% in PBS) and evaluated with a fluorescence microscope (450 nm–490 nm). The percentage of infected MΦ (infection rate) and the number of parasites/100 *infected* MΦ (mean ± SEM of four observations on two cultures; 300–400 MΦ observed) were determined. From these values the mean number of parasites/100 MΦ of the culture (= [infection rate : 100] × mean number of parasites/100 *infected* MΦ) was calculated.

2.5 Presentation of results and statistical analysis

The results in the figures and tables are expressed as mean ± SEM. Differences between treated and control MΦ cultures were tested for statistical significance by Student's *t*-test for unpaired samples (two tailed).

3 Results and discussion

3.1 TNF-α alone fails to activate MΦ for elimination of *L. major* AM

In a first set of experiments, we analyzed the effect of murine rTNF-α on the infection of adherent peritoneal MΦ cultures with *L. major* AM. Pilot experiments showed that TNF-α alone did not promote the elimination of intracellular AM at any of the dosages used (25 000, 2500, 250, 25, 2.5, 0.25, 0.025 U/ml). Although the MΦ monolayers were incubated with medium containing TNF-α prior to and throughout the infection, we never observed a significant reduction of the initial infection rate or of the parasite number/100 infected MΦ as compared to the medium control at any of the time points after infection (3, 24, 48, 72, 96 h; data not shown). In 5 out 12 experiments treatment of MΦ with TNF-α even caused an increased parasite burden of the MΦ cultures after 48 and 72 h, the reason for which remains unknown so far. From these data we conclude that TNF-α lacks the capacity to activate MΦ for killing of *L. major* AM in the absence of co-stimulatory signals. This result is in good accordance with earlier studies using *Trypanosoma cruzi* trypomastigotes for infection of resident peritoneal MΦ [20], in which TNF-α alone was also found to be ineffective. On the other hand, Bermudez and Young [21] found that TNF-α increased the killing of *Mycobacterium avium* by human monocyte-derived MΦ. Also, De Titto et al. [18] reported that TNF-α inhibits the multiplication of *T. cruzi* trypomastigotes in murine MΦ, whereas the intracellular replication of *Toxoplasma gondii* tachyzoites or bradyzoites is not affected by TNF-α. The effect of TNF-α therefore appears to depend on both the infecting organism and the source of MΦ used.

3.2 TNF-α induces parasite elimination in the presence of suboptimal dosages of IFN-γ

In other *in vitro* infection experiments it has been shown that TNF-α enhances MΦ destruction of intracellular pathogens in the presence of IFN-γ [19] or LPS [20]. We therefore investigated whether TNF acts synergistically with a suboptimal dose of IFN-γ (5 or 10 U/ml) or LPS (10 ng/ml) to induce MΦ killing of *L. major* AM. As demonstrated in Fig. 1, in the presence of IFN-γ (Fig. 1 a) or LPS (Fig. 1 b) the stimulation of MΦ monolayers with TNF-α (2500 U/ml) prior to and throughout the infection with AM led to a highly significant reduction of the parasite load as compared to the medium control. This was due to a decrease of both the infection rate and the average number of parasites per infected MΦ (data not shown). LPS or each of the cytokines alone were totally inactive in this respect (Fig. 1).

The synergistic anti-parasitic effect of TNF-α and IFN-γ was dependent on the concentration of TNF-α. Using 5 U/ml IFN-γ, the lowest effective dose of TNF-α was 2.5 U/ml (Table 1).

Higher dosages of IFN-γ (20 or 100 U/ml) promoted parasite elimination without any co-stimulation; however,

Table 1. Effect of TNF-α on the elimination of AM by MΦ in the presence of a constant dose of IFN-γ[a)]

Hours after infection	IFN-γ (5 U/ml) + TNF-α (U/ml) 250	25	2.5	0.25	IFN-γ (U/ml) 5	TNF-α (U/ml) 250[b)]	Medium
3	602 (± 39)	611 (± 42)	576 (± 41)	561 (± 27)	561 (± 24)	625 (± 36)	580 (± 22)
24	910 (± 63)	941 (± 46)	865 (± 55)	902 (± 43)	855 (± 44)	930 (± 51)	865 (± 37)
48	281* (± 19)	342* (± 29)	403* (± 14)	701 (± 26)	751 (± 32)	769 (± 33)	758 (± 22)
72	180* (± 13)	288* (± 16)	308* (± 17)	644 (± 24)	699 (± 49)	780 (± 41)	735 (± 51)

a) Adherent MΦ monolayers (2×10^5/chamber) were infected with a 5-fold excess of AM for 3 h and stimulated with 5 U/ml IFN-γ and various concentrations of TNF-α throughout the culture period. Control cultures were set up with each of the cytokines alone or in medium. The numbers indicate the mean number of parasites/100 MΦ (± SEM) which was determined as described in the legend to Fig. 1.

b) The results obtained with 25, 2.5 or 0.25 U/ml TNF-α alone were not statistically different and were therefore omitted in the table for the sake of brevity.

* Significantly different from controls with TNF-α alone ($p < 0.01$).

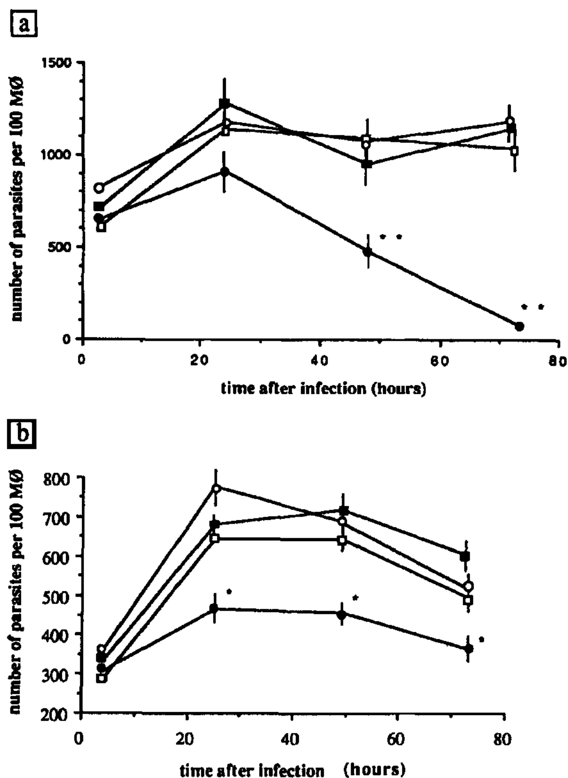

Figure 1. Effect of TNF-α on the elimination of AM by MΦ in the presence of IFN-γ or LPS. Adherent MΦ monolayers (2×10^5/chamber) were infected with a 5-fold excess of AM for 3 h and stimulated with TNF-α (2500 U/ml; ■) throughout the culture period in the presence of (a) IFN-γ (5 or 10 U/ml; ●) or (b) LPS (10 ng/ml; ●). Control cultures were stimulated with equal concentrations of either TNF-α or IFN-γ (○) or LPS (○) or were set up in medium alone (□). At the time points indicated the mean number of parasites/100 MΦ (± SEM) was determined as described in Sect. 2.5. For the sake of clarity the results for the cultures with 5 U/ml IFN-γ are omitted, as they did not differ from those of cultures stimulated with 10 U/ml IFN-γ. * Significant as compared to all controls ($p < 0.01$); ** highly significant as compared to all controls ($p < 0.001$).

TNF-α (2500 U/ml) still enhanced the effect of IFN-γ in a significant manner (Table 2). The synergistic anti-parasitic effect of TNF-α and IFN-γ was not caused by LPS contamination in either of the cytokine preparation, because the LPS concentrations in the stock preparations of the cytokines (< 0.025 ng/ml) were inactive and even lower than those in the culture medium itself (< 0.6 ng/ml). The observed anti-parasitic action of TNF-α and IFN-γ correlates with the finding that TNF-α and INF-γ synergistically stimulate MΦ for the production of reactive oxygen and nitrogen intermediates [25], which are involved in the killing process of *L. major* parasites [4, 26]. It is conceivable that the synergism is based on the well-established capacity of IFN-γ to induce the expression of TNF-α receptors [27–29], which are also found on MΦ [30].

3.3 Co-stimulation of MΦ with TNF-α and IL 4 does not cause elimination of intracellular parasites

IL 4 appears to be associated with the progression of the disease in murine *L. major* infections [10, 31]. On the other hand, MΦ-activating and resistance-mediating functions of IL 4 have also been reported [9, 32, 33], which prompted us to investigate the function of IL 4 in combination with TNF-α. When MΦ monolayers were incubated with medium containing IL 4 alone (280, 28 or 2.8 U/ml) prior to and throughout the infection with AM, we could neither observe a significant reduction nor an increase of the parasite burden of the MΦ (data not shown). IL 4 (280, 28, 2.8 U/ml) in combination with TNF-α (2500 U/ml) did not induce killing of *L. major* AM, but rather enhanced the survival of intracellular parasites as compared to IL 4 or TNF-α alone or the medium control (Fig. 2). These results illustrate that TNF-α in combination with IL 4 fails to exert a parasite-eliminating effect.

4 Concluding remarks

The study presented here provides the first evidence that TNF-α in combination with suboptimal dosages of IFN-γ induces killing of *L. major* AM by MΦ. TNF-α alone or in

Table 2. Effect of INF-γ on the elimination of AM by MΦ in the presence of a constant dose of TNF-α[a)]

Hours after infection	IFN-γ (U/ml) 5	20	100	TNF-α (2500 U/ml) + IFN-γ (U/ml) 5	20	100	TNF-α (U/ml) 2500	Medium
3	412 (± 38)	400 (± 31	316 (± 25)	395 (± 17)	471 (± 20)	407 (± 21)	318 (± 28)	325 (± 24)
24	592 (± 43)	639 (± 42)	485 (± 19)	518 (± 22)	588 (± 27)	535 (± 33)	667 (± 39)	644 (± 37)
48	603 (± 37)	614 (± 35)	499 (± 27)	376* (± 17)	147* (± 8)	117* (± 10)	703 (± 33)	627 (± 41)
72	581 (± 21)	157 (± 7)	123 (± 9)	30* (± 2)	≤ 1	0	588 (± 29)	480 (± 34)

a) Adherent MΦ monolayers (2×10^5/chamber) were infected with a 5-fold excess of AM for 3 h and stimulated with 2500 U/ml TNF-α and various concentrations of IFN-γ throughout the culture period. Control cultures were set up with TNF-α or INF-γ alone or in medium. The numbers indicate the mean number of parasites/100 MΦ (± SEM) which was determined as described in the legend to Fig. 1.

* See Fig. 1.

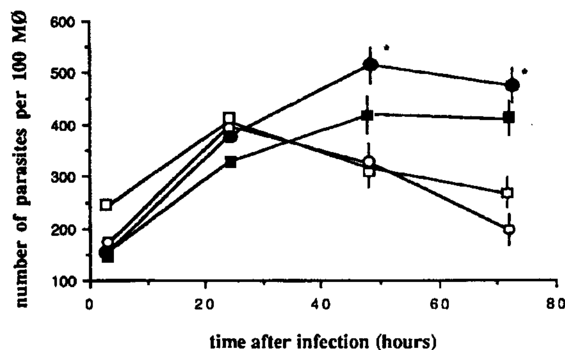

Figure 2. Effect of TNF-α on the elimination of AM by MΦ in the presence of IL 4. Adherent MΦ monolayers (2×10^5/chamber) were infected with a 5-fold excess of AM for 3 h and stimulated with TNF-α (2500 U/ml; ●) and IL 4 (28 U/ml; ●) throughout the culture period. Control cultures received either TNF-α (■) or IL 4 (○) or were set up in medium alone (□). The cultures were evaluated as described in the legend to Fig. 1. * Significant as compared to medium and IL 4 alone ($p < 0.01$).

combination with IL 4 was inactive in this respect. These findings have important implications for the understanding of the role of TNF-α in the pathogenesis of murine *L. major* infections. Recently, it has been demonstrated that in murine *L. major* infections resistance correlates with the expansion of IFN-γ-secreting $CD4^+$ T lymphocytes, whereas disease susceptibility is associated with an increased number of IL 4-producing $CD4^+$ T lymphocytes and a lack of IFN-γ-producing $CD4^+$ T lymphocytes [10]. On the other hand, a much higher LPS-induced TNF-α response is elicited in *L. major*-infected susceptible BALB/c mice than in resistant C57BL/6 mice (H. Moll et al., *Parasite Immunol.*, in press), which most likely is the consequence of an increased proportion of MΦ in their lymphoid organs throughout the infection [34]. In the light of our present results, TNF-α is unlikely to activate MΦ for the elimination of parasites in *L. major*-infected susceptible BALB/c mice, because they fail to provide sufficient amounts of IFN-γ as a co-stimulatory signal as opposed to what occurs in resistant C57BL/6 mice ([10]; H. Moll et al., manuscript submitted). TNF-α *per se* does not appear to be a disease-promoting lymphokine. However, in the absence of IFN-γ and the presence of IL 4, TNF-α may promote parasite survival. This process could even be enhanced by granulocyte-monocyte CSF, which is known to be induced by TNF-α [35, 36] and causes expansion of non-activated MΦ/monocytes serving as "safe targets" for Leishmania parasites [34, 37]. In summary, our results represent a further example of functional synergism of TNF-α and IFN-γ [19, 38-40], which may be of general relevance for the protection against intracellular parasites.

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