

Granuloma Necrosis during *Mycobacterium avium* Infection Does Not Require Tumor Necrosis Factor

Manuela Flórido¹ and Rui Appelberg^{1,2*}

Laboratory of Microbiology and Immunology of Infection, Institute for Molecular and Cell Biology,¹ and Instituto de Ciências Biomédicas de Abel Salazar,² University of Porto, Porto, Portugal

Received 27 April 2004/Returned for modification 10 June 2004/Accepted 30 June 2004

The infection of tumor necrosis factor (TNF)-deficient mice with low doses of the virulent *Mycobacterium avium* strain 25291 led to the appearance of necrotic granulomas at 93 days of infection, i.e., sooner than necrotic granulomas appeared in C57BL/6 animals. Additionally, TNF-deficient mice exhibited higher mycobacterial loads in the infected organs, had extremely exacerbated gamma interferon responses as evaluated in the sera of infected animals, and showed reduced survival. Thus, TNF is not required for granuloma necrosis.

One of the features of tuberculosis and other mycobacterial infections is the development of necrotic lesions originating in the granulomas induced by the infecting pathogens. These lesions are characterized by their macroscopic appearance and their microscopic structure. Starting as confluent masses of cells organized around a core of macrophages and epithelioid cells and surrounded by lymphocytes and occasional polymorphs, these lesions increase in size and may evolve to show areas of central necrosis which later progress into large areas of amorphous acellular material showing a cheesy (caseous) consistency upon macroscopic examination. Although the active immunological basis of the necrosis of granulomas is still disputable, a mouse model using virulent *Mycobacterium avium* has been developed that is amenable to extensive immunological studies regarding the mechanisms involved (2, 6). Previous work with such a system has highlighted the role of T cells in the induction of necrosis. Mice defective in either the $\alpha\beta$ T-cell receptor or the CD4 molecule did not develop necrosis (4, 6). The fact that interleukin-12 p40 and gamma interferon (IFN- γ) were also absolute requirements suggested that *M. avium*-induced caseous necrosis is dependent upon the activity of Th1 cells (4, 6). The lack of necrosis in the immunodeficient animals was not due to inadequate development of the granuloma, since the late administration of blocking monoclonal antibodies, specific for the CD4, IFN- γ , or interleukin-12 p40 molecules, prevented the necrosis of well-established and -developed granulomas (6). The relevance of these data extends beyond basic understanding of this phenomenon, as extensive Th1 polarization, namely during vaccination, may promote pathology that could hinder the protective efficacy of new-generation vaccines (7, 9). Here we assessed the possible involvement of tumor necrosis factor (TNF) in the necrosis of *M. avium* granulomas, since TNF is endowed with multiple proinflammatory activities and may cause the death of many cell types by apoptosis. The increased production of TNF has, in other systems, led to the exacerbation of pathology during mycobacterial infections. TNF production is closely related to

the severity of mycobacterial meningitis in rabbits (11), to the presence of cavitary tuberculosis in human patients (10), and to pathology in leprosy reversal reactions (8). The administration of TNF into mycobacterial lesions induced necrosis (1).

C57BL/6 mice were purchased from Harlan Iberica, and B6.TNF^{-/-} mice were bred at the Institute for Molecular and Cell Biology, Porto, Portugal, after breeders were purchased from B&K Universal (East Yorkshire, United Kingdom). Mice were infected intravenously with approximately 100 CFU of the highly virulent *M. avium* strain ATCC 25291, as described previously (6). The health of the mice was monitored by periodic inspection of the animals, as TNF-deficient animals are particularly susceptible to infection by mycobacteria (12). An initial experiment was performed to evaluate the mean survival time for TNF-deficient mice infected as indicated above and showed that these animals died between day 89 and day 102 of infection, with a mean survival time of 94 days ($n = 10$ mice). In an independent experiment, mice were infected, and at day 93, when some of the TNF-deficient mice started to look ill (i.e., had reduced weight, showed ruffled fur, and arched their backs while reducing their motor activity), they were sacrificed and the organs were collected for analysis. Sera were used for IFN- γ detection by enzyme-linked immunosorbent assay techniques as described previously (6). Granulomas in the liver were analyzed because most of the inoculum is trapped in this organ after an intravenous infection. Also, the granulomas and the surrounding parenchyma can be easily distinguished in the liver, in contrast to analysis of the spleen. To score for necrosis, the livers were initially scored macroscopically for the existence of tubercles which, when present, were processed for histology by fixing serial sections in formaldehyde, embedding them in paraffin, and staining them with hematoxylin and eosin. The lesions were then studied for the presence of necrosis.

At 3 months of infection, mice deficient in TNF became ill and were therefore sacrificed with a cohort of C57BL/6 controls at day 93 of infection. The development of necrosis at this time point was still modest in the controls, with only one animal in six exhibiting necrotic granulomas. In five of the C57BL/6 mice, granulomas exhibited no necrosis, although they were well developed in many cases (Fig. 1, top). In one animal, necrotic granulomas were detected (data not shown). In contrast, all six B6.TNF^{-/-} mice studied had necrotic gran-

* Corresponding author. Mailing address: Laboratory of Microbiology and Immunology of Infection, Institute for Molecular and Cell Biology, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Phone: 351.226074952. Fax: 351.226099157. E-mail: rappelb@ibmc.up.pt.

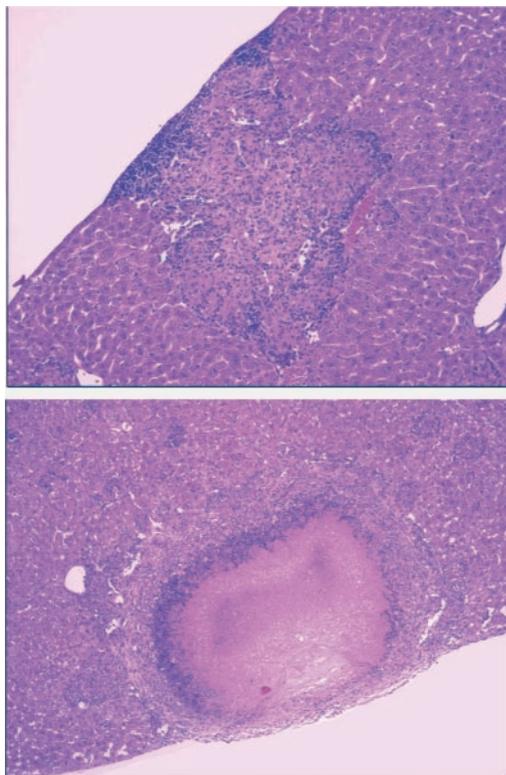


FIG. 1. Histological analysis of representative hematoxylin-eosin-stained sections from livers of infected animals at 93 days of infection. Top, a typical nonnecrotic lesion from C57BL/6 mice; bottom, a typical necrotic lesion found in TNF-deficient mice.

ulomas such as the one shown in Fig. 1 (bottom). The increased development of necrosis in TNF-deficient mice was associated with enhanced susceptibility to infection (Table 1). B6.TNF^{-/-} mice had particularly exacerbated infection in their livers and lungs (approximately 700- and 3,700-fold, respectively). Although the immunodeficient mice died with a mean survival time of about 3 months, control C57BL/6 mice survived up to 7 months when they had even higher mycobacterial burdens. A group of animals from the same experiment were sacrificed at day 219, and the mean bacterial counts (\pm standard deviations) were 10.3 ± 0.2 log CFU in the spleen, 10.6 ± 0.2 log CFU in the liver, and 9.8 ± 0.3 log CFU in the lungs.

TNF-deficient mice infected with *M. avium* 25291 had higher levels of IFN- γ in their sera than did the control infected mice

TABLE 1. Bacterial loads in organs of mice after infection with *M. avium* 25291^a

Organ	Log ₁₀ CFU/organ (mean \pm SD) ^b	
	C57BL/6	B6.TNF ^{-/-}
Spleen	6.71 \pm 0.33	8.26 \pm 0.27*
Liver	6.29 \pm 0.39	9.13 \pm 0.25*
Lung	5.00 \pm 0.36	8.57 \pm 0.44*

^a Mice were infected for 93 days with 100 CFU.

^b Values are means for six mice in each group. *, statistically significant difference in values for C57BL/6 and B6.TNF^{-/-} mice ($P < 0.001$, according to Student's *t* test).

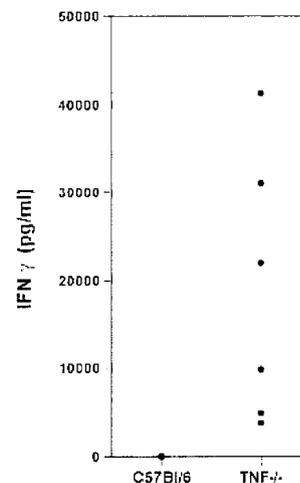


FIG. 2. Levels of IFN- γ detected in the sera of infected mice at 93 days of infection. Each symbol represents the mean of duplicate determinations for a single mouse. The difference in cytokine levels was statistically significant ($P < 0.01$, according to Student's *t* test).

(Fig. 2). The cytokine was detectable in only one of the C57BL/6 mice and at a very low level (65 pg/ml). In contrast, the concentration of IFN- γ in the sera of the TNF-deficient mice ranged from 3,776 to 41,234 pg/ml (Fig. 2).

Our study shows that granuloma necrosis induced by the virulent *M. avium* strain 25291 occurs in the absence of TNF. In fact, the incidence of necrotic granulomas was higher in the immunodeficient group than in the controls at the time point studied. The enhanced susceptibility of TNF-deficient mice to the mycobacterial infections precluded the study of longer-term infections. Although the acceleration of the induction of pathology was associated with enhanced mycobacterial loads, this effect on pathology was most likely due to the exacerbation of the immune response, namely of the IFN- γ response. It has already been shown that TNF, most likely acting through the type 1 receptor (TNF receptor super family 1a), exerts a down-modulatory effect on the immune response to mycobacteria (3, 5, 12). On the other hand, IFN- γ is a major element in the cascade of events that leads to necrosis in established granulomas (4, 6). However, we cannot exclude the fact that increased bacterial loads may contribute to the acceleration of the necrosis of granulomas in TNF-deficient mice.

In summary, we have shown here that TNF is not required for the development of caseous necrosis of mycobacterial granulomas. In addition, we have shown that TNF acts to down-regulate the Th1 response that is triggered by the mycobacterial infection, as previously recognized (3, 5, 12). The hypertrophic immune response in the absence of TNF may, in fact, accelerate the appearance of granuloma necrosis.

This work was supported through grants from the Fundação para a Ciência e a Tecnologia (POCTI/32629) and the Fundação Calouste Gulbenkian (SDH.IC.I.01.15). M.F. is a fellow from FCT.

We are indebted to Alexandra Rêma, Fátima Faria, and Célia Lopes for their technical help.

REFERENCES

1. Al Attiyah, R., H. Rosen, and G. A. W. Rook. 1992. A model for the investigation of factors influencing haemorrhagic necrosis mediated by tumour necrosis factor in tissue sites primed with mycobacterial antigen preparations. *Clin. Exp. Immunol.* **88**:537-542.

2. **Benini, J., E. M. Ehlers, and S. Ehlers.** 1999. Different types of pulmonary granuloma necrosis in immunocompetent vs. TNFRp55-gene-deficient mice aerogenically infected with highly virulent *Mycobacterium avium*. *J. Pathol.* **189**:127–137.
3. **Ehlers, S., J. Benini, S. Kutsch, R. Endres, E. T. Rietschel, and K. Pfeffer.** 1999. Fatal granuloma necrosis without exacerbated mycobacterial growth in tumor necrosis factor receptor p55 gene-deficient mice intravenously infected with *Mycobacterium avium*. *Infect. Immun.* **67**:3571–3579.
4. **Ehlers, S., J. Benini, H.-D. Held, C. Roeck, G. Alber, and S. Uhlig.** 2001. $\alpha\beta$ T cell receptor-positive and interferon- γ , but not inducible nitric oxide synthase, are critical for granuloma necrosis in a mouse model of mycobacteria-induced pulmonary immunopathology. *J. Exp. Med.* **194**:1847–1859.
5. **Ehlers, S., S. Kutsch, E. M. Ehlers, J. Benini, and K. Pfeffer.** 2000. Lethal granuloma disintegration in mycobacteria-infected TNFRp55 $^{-/-}$ mice is dependent on T cells and IL-12. *J. Immunol.* **165**:483–492.
6. **Flórido, M., A. M. Cooper, and R. Appelberg.** 2002. Immunological basis of the development of necrotic lesions following *Mycobacterium avium* infection. *Immunology* **106**:590–601.
7. **Gonzalez-Juarrero, M., J. Turner, R. J. Basaraba, J. T. Belisle, and I. M. Orme.** 2002. Florid pulmonary inflammatory responses in mice vaccinated with Antigen-85 pulsed dendritic cells and challenged by aerosol with *Mycobacterium tuberculosis*. *Cell. Immunol.* **220**:13–19.
8. **Khanolkar-Young, S., N. Rayment, P. M. Brickell, D. R. Katz, S. Vinayakumar, M. J. Colston, and D. N. J. Lockwood.** 1995. Tumour necrosis factor-alpha (TNF- α) synthesis is associated with the skin and peripheral nerve pathology of leprosy reversal reactions. *Clin. Exp. Immunol.* **99**:196–202.
9. **Taylor, J. L., O. C. Turner, R. J. Basaraba, J. T. Belisle, K. Huygen, and I. M. Orme.** 2003. Pulmonary necrosis resulting from DNA vaccination against tuberculosis. *Infect. Immun.* **71**:2192–2198.
10. **Tsao, T. C. Y., J. Hong, L. Li, M. Hsieh, S. Liao, and K. S. S. Chang.** 2000. Imbalances between tumor necrosis factor- α and its soluble receptor forms, and interleukin-1 β and interleukin-1 receptor antagonist in BAL fluid of cavitary pulmonary tuberculosis. *Chest* **117**:103–109.
11. **Tsenova, L., A. Bergtold, V. H. Freedman, R. A. Young, and G. Kaplan.** 1999. Tumor necrosis factor α is a determinant of pathogenesis and disease progression in mycobacterial infection in the central nervous system. *Proc. Natl. Acad. Sci. USA* **96**:5657–5662.
12. **Zganiacz, A., M. Santosuosso, J. Wang, T. Yang, L. Chen, M. Anzulovic, S. Alexander, B. Gicquel, Y. Wan, J. Bramson, M. Inman, and Z. Xing.** 2004. TNF- α is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. *J. Clin. Investig.* **113**:401–413.

Editor: V. J. DiRita