BRIEF COMMUNICATION

Impact of tumor necrosis factor receptor p55 deficiency in susceptibility of C57BL/6 mice to infection with *Leishmania (Leishmania) amazonensis*

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Tumor necrosis factor (TNF) is involved in host resistance to several intracellular pathogens. Although the critical role of TNF receptor (TNFR)p55 in *Leishmania (Leishmania) major* infection has been demonstrated, the impact of TNFRp55 deficiency on *L. (L.) amazonensis* infection has not been explored. *L. (L.) amazonensis*-infected TNFRp55$^{-/-}$ mice failed to resolve lesions, whereas C57BL/6 wild-type mice completely healed. The susceptibility of the TNFRp55$^{-/-}$ mice was characterized by higher lesion size and histopathological damage in comparison with the wild-type mice. A marked increased of the splenic index was observed

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TNFRp55$^{-/-}$;
Wild type

in the TNFRp55$^{-/-}$ mice after 15 weeks infection. These results show that in the absence of TNFRp55, *L. (L.) amazonensis*-infected knockout mice fail to resolve lesions, whereas wild-type mice completely heal.

Leishmaniasis, a parasitic disease caused by protozoa of the genus *Leishmania*, is an important endemic disease affecting > 12 million people worldwide. Different species of *Leishmania* cause disease in different areas of the world. Cutaneous leishmaniasis may be caused by *Leishmania (Leishmania) major* in the Old World and by *Leishmania (L.) amazonensis* or *Leishmania (Viannia) braziliensis* in the New World, including Argentina.[1]

It has been determined that different *Leishmania* species elicit distinctive patterns of the immune response in animal models. This can be illustrated by the different susceptibility of CBA mice, which are known to be resistant to *L. (L.) major* but susceptible to *L. (L.) amazonensis* infection. Infection of CBA mice with *L. (L.) major* generates interferon (IFN)-γ, which is sufficient to prevent the development of a T helper (Th)2 response and to induce instead a protective Th1 immune response. On the contrary, CBA mice produce high levels of interleukin-4 but they do not produce significant amounts of IFN-γ after infection with *L. (L.) amazonensis*. The lack of IFN-γ biases the immune response towards a Th2 profile.[2]

The effect of tumor necrosis factor (TNF) in the outcome of *Leishmania* infection has been more thoroughly studied in C57BL/6 (B6) mice deficient in the TNF receptor (TNFR) p55 (TNFRp55$^{-/-}$) and TNFRp75 (TNFRp75$^{-/-}$) compared with the wild-type (WT) mice of the same genetic background[3,4] and in mice deficient in the production of soluble TNF infected with *L. (L.) major*.[5]

Vieira et al[5] have demonstrated that both TNFRp55$^{-/-}$ and WT mice develop a normal Th1 type response and are able to eliminate parasites *in vivo* during *L. (L.) major* infection. These results demonstrate that the macrophage microbicidal activity is not dependent on the p55 receptor for TNF; however, TNFRp55$^{-/-}$ mice develop larger lesions.

In another experimental model, B6 knockout (KO) mice infected with *L. (L.) major* developed a visceral form in which the parasite spread rapidly, resulting in death of the mice at 6–9 weeks post-infection.[3] Although the immune response in both strains was Th1 type, the response of KO mice was delayed with respect to that of the WT. There was a remarkable short survival in the KO animals, which was even shorter than the usual in BALB/c mice infected with the same strain, indicating the high susceptibility of the KO mice to infection. However, when the same authors used a different strain of *L. (L.) major* named FRIEDLIN, the results were different. Mice infected with *L. (L.) major* FRIEDLIN strain exhibited partial resistance characterized by chronic, nonhealing skin lesions without lethality.[6]

Although the relevance of TNFRp55 in *L. (L.) major* infection has been studied, the impact of TNFRp55 deficiency on *L. (L.) amazonensis* infection has not been explored. For this reason, the purpose of the present work was to evaluate the susceptibility of TNFRp55$^{-/-}$ B6 mice to *L. (L.) amazonensis* infection compared to B6 WT mice. To perform these studies, the TNFRp55$^{-/-}$ mice were obtained from the Max von Pettenkofer Institute, Munich, Germany. Mice were kept under specific pathogen-free conditions in a positive pressure cabinet (EHRET, Emmendingen, Germany) and provided with sterile food and water *ad libitum*. Three independent experiments were carried out with five mice per group and all experiments involving animals were approved by the Institutional Review Board for Animal Experimentation of the School Medicine from the National University of Cuyo, Mendoza, Argentina (IACUC No. 18/2013). Differences among groups were tested for significance by two-way analysis of variance followed by Bonferroni post-test, and by Student's unpaired *t* test using GraphPad Prism for Windows version 5.01 (GraphPad Software, San Diego, CA, USA).

The strain of *L. (L.) amazonensis* (MHOM/VE/84/MEL) used in this study was kindly provided by Dr. Miguel Angel Basombrio, from the Experimental Pathology Institute from the National University of Salta, Salta, Argentina. Promastigotes of *L. (L.) amazonensis* were grown as described.[7] An inoculum of 1×10^6 promastigotes of *L. (L.) amazonensis* per 50 μL phosphate-buffered saline was injected into the right hind footpad in 6–8-week-old mice TNFRp55$^{-/-}$ on a B6 background and B6 WT mice. The course of cutaneous lesions development was analyzed and compared during 15 weeks. As can be seen in Fig. 1A, the WT mice developed lesions at the site of infection, which peaked in size between Week 6 and Week 10, and then steadily receded until they disappeared at the end of the protocol. By contrast, TNFRp55$^{-/-}$ mice developed larger, ulcerated, and ceraceous lesions and swollen toes. Furthermore, hind leg retraction and hip and hinge joint deterioration were evident. Lesions did not heal and worsened dramatically over time, leading to loss of the infected foot. Due to the sharp deterioration of the infected footpad in all mice of the TNFRp55$^{-/-}$ group, animals had to be euthanized at Week 13 and the last two measurements of the footpad swelling could not be completed.

For histological evaluation of the lesions induced by inoculation of *L. (L.) amazonensis* in WT and TNFRp55$^{-/-}$ mice, fragments of footpad lesions were fixed in Bouin's solution and embedded in paraffin. Tissue sections of 5–6 μm were cut, mounted onto glass slides, and stained with hematoxylin/eosin. Images were taken with a Nikon Eclipse E200 Microscope (Nikon, Tokyo, Japan) fitted with a digital still camera Micrometric SE Premium (Nikon). Histological damage was assessed by observation of 10 different fields (40 × magnification) of hematoxylin/eosin-stained sections from each animal.

Histological examination of the lesions in both groups revealed similar histopathological features at least until
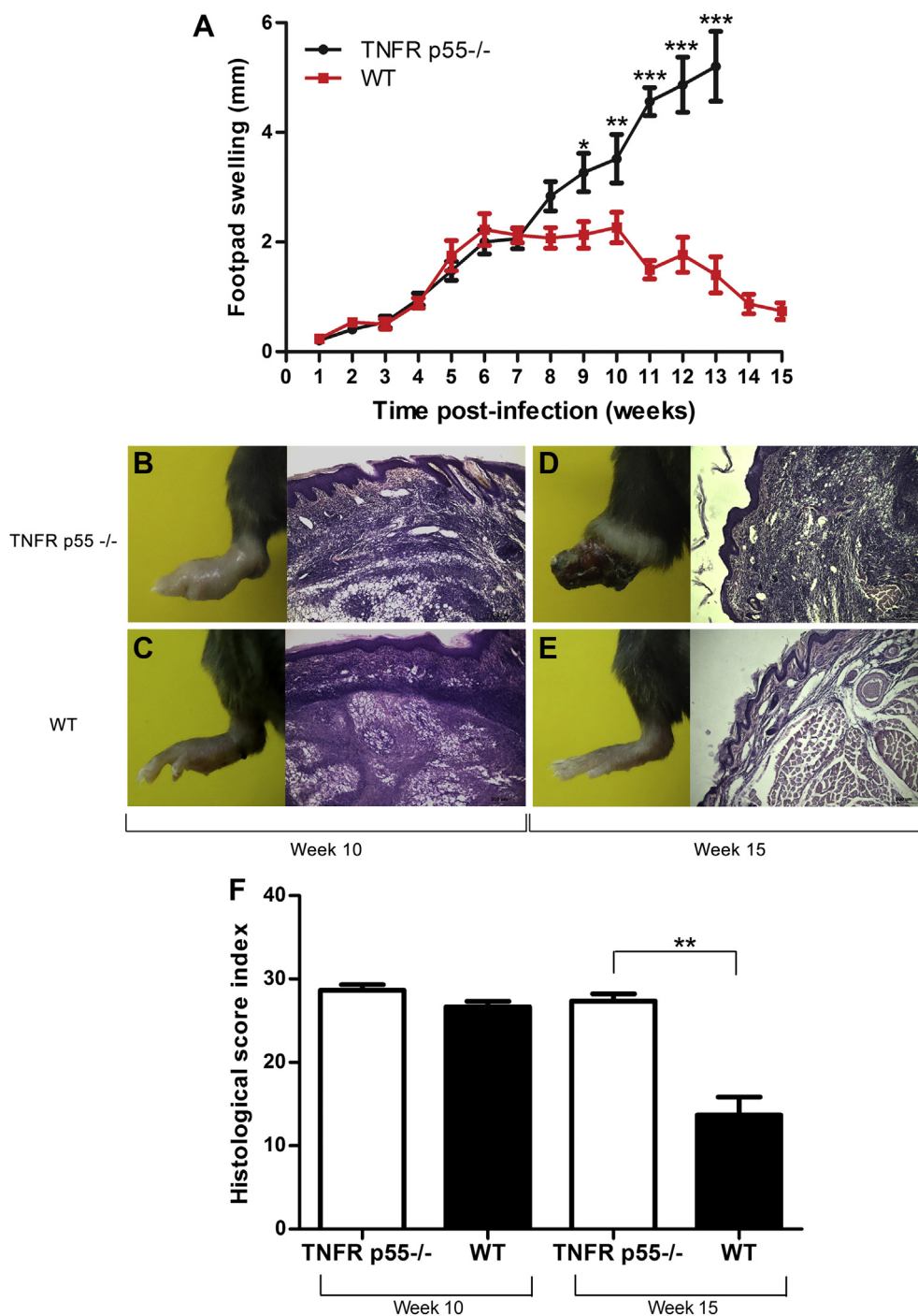
Figure 1. Lesions and histological evaluation in TNFR p55$^{-/-}$ and WT mice infected with *L. (L.) amazonensis*. Mice were infected with 1×10^6 promastigotes of *L. (L.) amazonensis* in the footpads. Footpad lesion development was followed by weekly measurement of footpad swelling using a digital caliper and the value for uninfected mice was subtracted from each infected footpad to estimate lesion size (A). Macroscopic and histological aspects from TNFRp55$^{-/-}$ and WT footpad, infected with *L. (L.) amazonensis* (B and C) 10 weeks and (D and E) 15 weeks after infection. Histological score index of the footpad infection site from TNFRp55$^{-/-}$ and WT lesions (F) 10 weeks and 15 weeks after infection ($n = 3$ mice per group). Data shown represent the mean values ± standard error of the mean of three independent experiments. The asterisks indicate significant differences: *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$. Data are from one experiment of three performed independently. TNFRp55 = tumor necrosis factor receptor p55; WT = wild-type.

Week 10, showing a strong inflammatory response characterized by severe inflammation with intense tissue destruction of the dermis and epidermis. Also, extensive collections of vacuolated and heavily parasitized macrophages were observed. Amastigote forms were attached to the wall of the parasitophorous vacuoles and a large number of parasitized macrophages and extensive areas of necrosis were seen. In these areas of necrosis, free amastigotes were seen among inflammatory cells. Occasionally, areas of necrosis were infiltrated by granulocytes, giving rise to micro-abscesses (Fig. 1A and C).

Fifteen weeks after infection, the histological examination of the lesions from the TNFRp55$^{-/-}$ group showed a large number of inflammatory cells with the same histopathological characteristics described above (Fig. 1D), whereas lesions from the WT mice (Fig. 1E) showed few inflammatory cells. Histological changes in the lesions were semiquantitatively graded based on the criteria described by Côrtes et al.[8] The histopathological scoring and grading system used to evaluate the degree of inflammation was as follows: 0: none; 1: slight infiltration of inflammatory cells; 2: moderate infiltration; 3: severe infiltration.[8] The total score was defined as the sum of all the scores. Each slide was scored by two independent observers and the average score was used. A significant decrease in the histological score index resulted from the analysis of the degree of inflammation in the footpad lesion of the WT group compared to those of the TNFRp55$^{-/-}$ group (Fig. 1F).

The splenic index was used as an indicator of disease progression to evaluate the degree of susceptibility of both experimental groups to *L. (L.) amazonensis* infection. Mice were weighed and sacrificed by cervical dislocation. The spleen was removed from sacrificed animals and weighed in a digital precision balance. This index was determined according to the formula[9]:

Splenic index = spleen weight/whole body weight × 100.

At 10 weeks post-infection, the splenic index was similar in both groups. However, at Week 15, the splenic index of the WT mice increased slightly, whereas that of the

TNFRp55$^{-/-}$ mice increased fivefold (Table 1). This result shows that TNFRp55$^{-/-}$ mice, experimentally infected with *L. (L.) amazonensis*, developed massive splenomegaly, which is a hallmark of visceral leishmaniasis. It is known that infection with *L. (L.) amazonensis* may be associated with many different clinical presentations including visceralization.[10]

The role of TNF in promoting resistance to intracellular parasitic pathogens such as *L. (L.) major* has been investigated in a range of experimental models,[3-6] but the impact of TNFRp55 deficiency on *L. (L.) amazonensis* infection has not been explored. In this work, we have shown that TNFRp55 plays a key role in the protection against leishmaniasis induced by *L. (L.) amazonensis* infection in B6 mice. These results are similar to those reported by Oliveira et al,[4] using infection of a similar mouse model with *L. (L.) major*. In both cases, the TNFRp55$^{-/-}$ mice failed to resolve lesions that were characterized by intense infiltration of inflammatory cells.

Taken together, our results demonstrate that TNFRp55 plays a critical role in the control of *L. (L.) amazonensis* pathogenicity. These results indicate that TNFRp55 plays a key role (1) in the clearance of the inflammatory infiltrate on the infected footpad; (2) in the development of splenomegaly associated with infection; and (3) in the healing process of the footpad lesions. These are the first data demonstrating that TNF is essential for the *in vivo* control of *L. (L.) amazonensis* infection.

To elucidate the mechanisms by which TNF exerts its protective role in this model, we are presently studying the consequences of TNF signaling on several different parameters of the immune response in infected mice.

Conflicts of interest

All authors declare no conflicts of interest.

Acknowledgments

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Table 1 Splenic indexes generated by the infection of *L. (L.) amazonensis* in WT and TNFRp55$^{-/-}$ mice[a]

	WT		TNFRp55−/−	
	Mean	Standard error	Mean	Standard error
Week 10	0.54	0.02	0.47*	0.02
Week 15	0.86	0.09	2.01**	0.31

[a] Mice were infected with *L .(L.) amazonensis* in the footpad (1×10^6) and after 10 weeks and 15 weeks of infection, animals were weighed, sacrificed, and the spleen collected. The splenic index was determined. Results represent the mean of five animals for each experimental group. Data shown represent the mean ± standard error of the mean of three independent experiments.
*$p = 0.108$ Student's *t* test.
**$p = 0.012$ Student's *t* test.
TNFRp55 = tumor necrosis factor receptor p55; WT = wild-type.

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