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Control of *Leishmania major* Infection in Mice Lacking TNF Receptors[1]

Michelle Nashleanas, Suzanne Kanaly, and Phillip Scott[2]

TNF participates in the induction of nitric oxide (NO) production and macrophage activation, leading to the elimination of intracellular pathogens. We previously found that TNF receptor p55-deficient mice (TNFRp55$^{-/-}$) control replication of *Leishmania major* in vivo but fail to resolve their lesions. Here we report that mice lacking the p75 receptor (TNFRp75$^{-/-}$) or both receptors (TNFRp55p75$^{-/-}$), also control parasite replication, albeit mice lacking the p55 receptor (either TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$) are delayed in their elimination of *L. major* compared with controls. All TNF receptor-deficient mice developed a Th1-type immune response and up-regulated inducible NO synthase (iNOS) mRNA gene expression in lesions during infection. Thus, neither TNF receptor appears to be absolutely required for NO production or elimination of *L. major* in vivo. In vitro, however, while macrophages from naive TNFRp75$^{-/-}$ mice could be activated to produce NO and kill *L. major*, we observed a defect in NO production and parasite killing by resident peritoneal macrophages from naive TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$ mice. However, when macrophages were elicited with leishmanial Ag from 4-wk-infected TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$ mice, they produced NO and were leishmanicidal. These data suggest that the TNFRp75 plays no essential role in *L. major* infection in mice and that the p55 receptor may be required for optimal macrophage activation. However, the results also show that a mechanism exists by which macrophages can be primed in vivo during *L. major* infection to produce NO and kill *L. major* in the absence of signaling through either of the TNF receptors. ***The Journal of Immunology*, 1998, 160: 5506–5513.**

Leishmania major is an intracellular protozoan parasite of macrophages that causes a wide range of diseases in man and animals. Immunologic control of *L. major* is dependent upon cell-mediated immunity, involving the production of IFN-γ at levels sufficient to activate macrophages to kill the parasites via induction of NO[3] production (1–3). Previous studies have shown that IFN-γ-activated macrophages infected in vitro with *L. major* require endogenously produced TNF for NO production and parasite killing (4). Consistent with a requirement for TNF are studies showing that in vivo neutralization of TNF using mAbs enhanced susceptibility to *L. major*, although such animals eventually healed (5, 6). However, since the long-term efficacy of TNF neutralization using mAb is problematic, these experiments left unresolved the issue of an absolute requirement for TNF in healing.

TNF, which includes homotrimeric TNF-α and lymphotoxin-α (also known as TNF-β) binds to two different receptors, known as the TNFRp55 or the TNFR1, and the TNFRp75 or the TNFR2 (7). These receptors have distinct cytoplasmic domains and mediate different functions. Studies with TNFR-deficient mice found that many of the responses associated with TNF are mediated through the TNFRp55, such as macrophage activation and sensitivity to endotoxin, and involve NF-κB activation (8, 9). Furthermore, the presence of the p55 receptor is required for resistance to *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Toxoplasma gondii* (8–11). In addition, the TNFRp55 also contains a death domain, homologous to the Fas death domain, and signaling through the p55 receptor can also induce apoptosis (12, 13). At present, the factors determining whether signaling through the TNFRp55 leads to NF-κB activation or the induction of apoptosis are not defined. Signaling through the p75 receptor can also be associated with NF-κB activation, although fewer functions are ascribed to this receptor (14, 15). Similar to wild-type controls, TNFRp75$^{-/-}$ are susceptible to endotoxin and resistant to *L. monocytogenes*. However, in contrast to control mice, they were found to be resistant to TNF-induced skin necrosis (14).

We previously reported that mice lacking the TNFRp55 were able to eliminate *L. major* parasites at the site of infection, although, interestingly, they did not heal their lesion (16). The ability of TNFRp55$^{-/-}$ mice to eliminate *L. major* parasites was unexpected, since, in the absence of the TNFRp55, macrophages do not produce NO in response to stimulation with IFN-γ and TNF-α (10, 16), suggesting that the TNFRp55 is required for this pathway of macrophage activation. However, it remained possible that in the absence of the p55 receptor, the p75 receptor might compensate for NO induction. Therefore, we examined the course of *L. major* infection and in vitro macrophage activation in TNFRp75$^{-/-}$ and doubly deficient mice generated by crossing the p55 and p75 deficient mice (TNFRp55p75$^{-/-}$). We found that the outcome of *L. major* infection is not significantly altered in the absence of the TNFRp75, but that mice lacking both receptors are unable to heal. Nevertheless, all of the mice developed a Th1-like response, and all eventually up-regulated iNOS within, and eliminated parasites from, their lesions. In contrast, we observed a defect in the activation of resident macrophages from mice lacking the p55 receptor (both TNFRp55p75$^{-/-}$ and TNFRp55$^{-/-}$). To determine whether a TNFR-independent mechanism for activation of macrophages is

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[3] Abbreviations used in this paper: NO, nitric oxide; iNOS, inducible nitric oxide synthase; HPRT, hypoxanthine-quanine phosphoribosyltransferase; wt, wild-type; SLA, soluble leishmanial Ag.

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invoked during *L. major* infection, we elicited peritoneal macrophages from *L. major*-infected mice with leishmanial Ag and studied their ability to kill *L. major* and produce NO. In contrast to resident macrophages from uninfected TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$ mice, macrophages specifically elicited during infection from both of these mouse strains were activated, demonstrating that *L. major* infection is associated with activation of macrophages in vivo in the absence of both TNF receptors.

Materials and Methods

Mice

Receptor-deficient and control mice were bred and housed at the University of Pennsylvania. Mice were used at 6 and 8 wk of age. TNFRp55 mice were backcrossed onto the C57BL/6 for seven generations (8). The TNFRp75$^{-/-}$ mice and TNFRp55p75$^{-/-}$ mice were maintained on a random C57BL/6 × 129 hybrid background and were initially provided by Dr. Mark Moore (Genentech, South San Francisco, CA) (14). Wild-type (+/+) littermates from the 7th backcross to C57BL/6 (wt (B6(7)) mice and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were maintained for use as controls for the TNFRp55-deficient animals. No significant differences between wt B6(7) and C57BL/6J (The Jackson Laboratory) were detected, and only data from B6(7) mice is shown. C57BL/6 × 129 hybrids (wt (129/B6)) were used as controls for the TNFRp75$^{-/-}$ and TNFRp55p75$^{-/-}$ mice.

Parasites and Ag

L. major (WHO MHOM/IL-1/80 Freidlin clone) was maintained in a log phase of growth in Grace's insect cell culture medium (Life Technologies, Grand Island, NY) supplemented with 20% FCS. Stationary phase promastigotes were selected for metacyclic stage organisms by *Arachis hypogae* agglutinin (Sigma, St. Louis, MO) as described (17). Mice were infected with 2 million metacyclic promastigotes in the hind footpad. Amastigotes for in vitro macrophage experiments were obtained from lesions of infected scid mice, and viable amastigotes were counted by fluorescein diacetate fluorescence as described (18). Soluble leishmanial Ag (SLA) was prepared as described (19).

Course of infection

Lesion size was determined by measuring the diameter of the footpad with a Starret dial caliper and subtracting the diameter of the contralateral uninfected footpad. The total number of parasites in lesions were determined by limiting dilution analysis as previously described (20).

Preparation of cells

Infected mice were sacrificed and the popliteal lymph nodes, spleen, and feet were collected. Lymph nodes and spleens were disrupted with a glass tissue homogenizer (Wheaton; Fisher, Pittsburgh, PA), and the resulting cell suspension was washed and subsequently resuspended in complete tissue culture medium (CTCM) (4.5 mg/ml glucose DMEM, 10% FCS, 25 mM HEPES, 5×10^{-5} β-2-ME (2 ME), 100 U/ml penicillin-6-potassium, 100 μg streptomycin sulfate, 2 mM glutamine). Ag specific recall responses were determined by stimulation of cells with SLA (50 μg/ml) and collection of supernatants at 72 h for IFN-γ and IL-4 analysis (19).

Peritoneal resident or Ag-elicited macrophages (peritoneal exudate cells, PECs) were harvested by peritoneal lavage, and incubated in polypropylene tubes in complete tissue culture medium without β2-ME. Resident macrophages are derived from naive mice that have not been injected i.p. with an inflammatory agent. In contrast, Ag-elicited macrophages are derived from uninfected or *L. major*-infected mice that were injected with 1 ml freeze/thawed *L. major* (the equivalent of 10 million parasites) into the peritoneal cavity and were harvested 4 days after injection of Ag. Cells were cultured with various combinations of the following: IFN-γ (Genentech), LPS (Sigma, L5014), and/or neutralizing mAb to TNF (XT22.11). Macrophages were infected with *L. major* amastigotes at a 2:1 ratio and incubated at 37°C for 2 h in suspension cultures (polypropylene tubes). Nonphagocytosed amastigotes were washed from the cultures by centrifugation, and the cells were resuspended in medium with cytokine and Ab. Aliquots were removed at 2 and 72 h and stained for visual quantitation of the infection. At 72 h supernatants were harvested and assayed for NO_2.

Cytokines, NO, and competitive PCR

IFN-γ and IL-4 were measured using specific two-site ELISAs as previously described (21). NO production was assessed by measuring NO_2 in supernatants harvested at 72 h using the Greiss reagent (22). The expression of iNOS mRNA was measured using a competitive RT-PCR as previously described (23). Briefly, footpads were harvested and immediately frozen in liquid nitrogen. The tissue was homogenized in GITC (guanidium isothiocyanate) lysis buffer, 1/10 volume of 2 M sodium acetate was added, then RNA was extracted using phenol-chloroform isoamyl alcohol (50:49:1). Samples were precipitated with an equal volume of isopropanol, and RNA concentration was calculated by OD at 260 nm. Two micrograms of RNA was reverse transcribed using Superscript 2 (Life Technologies) and random heximer primers (Promega, Madison, WI). PCR was done using a multiple-cytokine competimer construct (23). cDNA was normalized to 0.1 attamoles polycompetimer for hypoxanthine-quanine phosphoribosyltransferase (HPRT) (GTT GGA TAC AGG CCA GAC TTT GTT G and GAG GGT AGG CTG GCC TAT AGG CT). Using normalized cDNA, we performed competitive PCR using iNOS primers (TGG GAA TGG AGA CTG TCC CAG and GGG ATC TGA ATG TGA TGT TTG). A titration of the competimer was done to determine the point at which the amplification of the competimer and sample were equivalent by ethidium bromide staining. Results are expressed as the ratio of iNOS message to HPRT.

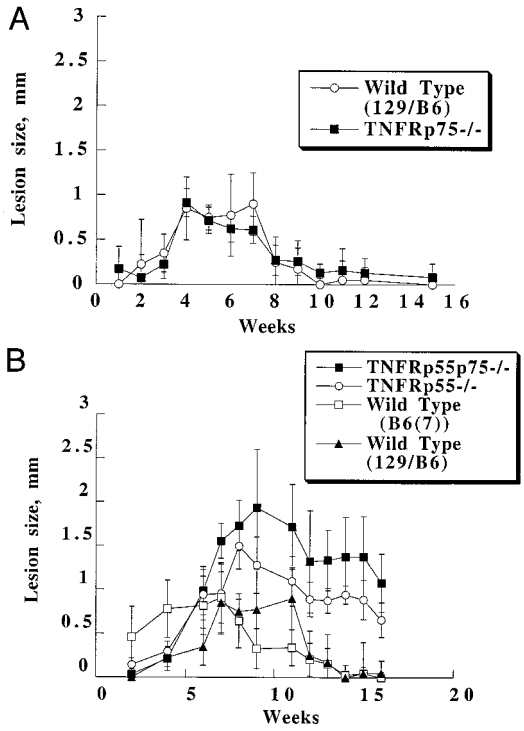

FIGURE 1. Course of *L. major* infection and control of parasites in TNFR-deficient mice. Mice were infected s.c. in the hind footpad with 2×10^6 metacyclic promastigotes. Lesion size was determined by measuring swelling in the infected footpad and subtracting from the contralateral uninfected footpad. *A*, Course of infection in TNFRp75$^{-/-}$compared with wt (129/B6) controls. TNFRp75$^{+/-}$ heterozygotes exhibited a course of infection identical to TNFRp75$^{-/-}$ and controls (data not shown). Similar results were obtained in two additional experiments. *B*, Course of infection in TNFRp55p75$^{-/-}$ mice compared with TNFRp55$^{-/-}$ and wt (129/B6 and B6/7) mice. Similar results were obtained in two additional experiments. Values shown represent mean ± SD of the footpad swelling.

Statistics

Results are expressed as the mean ± SD or SE as indicated in figure legends. Significance was determined by the Student *t* test, with $p < 0.05$ defining significance over control group.

Results

Course of L. major *infection in TNFR-deficient mice*

TNFRp55$^{-/-}$ mice fail to heal following *L. major* infection, so it was of interest to determine the course of infection in mice lacking

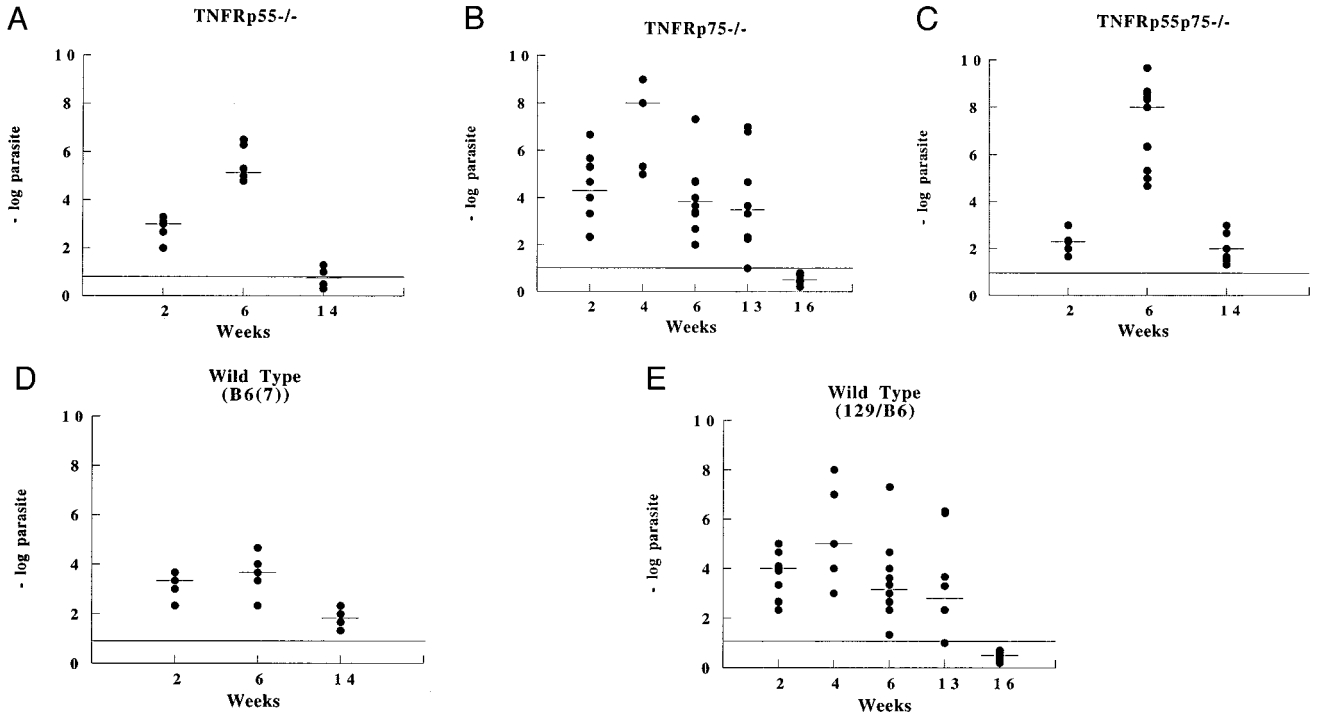

FIGURE 2. Control of *L. major* replication in TNFR-deficient mice. Mice were sacrificed at various times postinfection, and parasites were quantitated from the footpad by limiting dilution analysis. Each data point represents an individual animal, with horizontal bars representing median -log parasite number at the time point. Solid lines represent the limit of detection of the assay. *A*, TNFRp55$^{-/-}$ mice. *B*, TNFRp75$^{-/-}$ mice. *C*, TNFRp55p75$^{-/-}$ mice. *D*, wt (B6(7) mice. *E*, wt (129/B6) mice. Parasite counts were significantly higher at 6 wk postinfection in TNFRp55p75$^{-/-}$ and TNFRp55$^{-/-}$, compared with their wt controls ($p < 0.01$).

the TNFRp75 or both receptors. Therefore, TNFRp75$^{-/-}$ and TNFRp75$^{+/+}$ littermates were infected with *L. major*, and the course of infection was followed (Fig. 1*A*). No significant differences were seen in lesion sizes between control mice and those lacking the p75 TNF receptor. In contrast, TNFRp55p75$^{-/-}$ mice exhibited a nonhealing course of infection similar to that which we previously reported with the TNFRp55$^{-/-}$ mice (Fig. 1*B*).

Control of parasite replication in TNFR-deficient mice

Using limiting dilution analysis, we found no significant differences in the ability of TNFRp75$^{-/-}$ mice to eliminate parasites from the lesions, compared with their wt (129/B6) controls (Fig. 2, *B* and *E*). In contrast, TNFRp55p75$^{-/-}$ mice exhibited a delay in parasite clearance compared with their wt controls (Fig. 2, *C* and *E*), with a significantly higher parasite burden at 6 wk postinfection. Nevertheless, by 14 wk postinfection, most of the parasites had been eliminated from the lesions, demonstrating that neither TNF receptor is required for control of parasites. The results with the TNFRp55p75$^{-/-}$ mice are similar to those that we previously reported in TNFRp55$^{-/-}$ mice (16).

Development of Th1-type immune responses in TNFR-deficient mice

Resistance to *L. major* infection depends on the development of a Th1-like response, associated with production of IFN-γ but little IL-4 (24–27). Previous work has suggested that TNF may play a role in the ability of T cells to differentiate into Th1 cells (28). While our previous studies demonstrated that Th1 cells develop in the absence of the p55 TNF receptor, we were interested to determine whether Th1 cells could develop in the absence of both receptors. Therefore, we measured the levels of IFN-γ and IL-4 produced by the lymph node and spleen cells taken from mice infected for 2 and 14 wk. At all time points, IL-4 was below the level of detection (data not shown). In contrast, IFN-γ was produced by Ag-stimulated cells from control and TNFRp55p75$^{-/-}$, as well as TNFRp75$^{-/-}$ mice (Table I). Thus, like TNFRp55$^{-/-}$ mice, the TNFRp75$^{-/-}$ and double deficient mice develop a Th1 response, demonstrating that TNF signaling through either of these receptors is not required for Th1 cell development.

Table I. *IFN-γ production in TNFR-deficient mice*[a]

Mouse Genotype	IFN-γ (ng/ml)	
	2 wk	14 wk
wt (B6(7))		
Medium	<0.007	0.24 ± 0.61
SLA	10.4 ± 7.8	21.8 ± 10.2
TNFRp55$^{-/-}$		
Medium	<0.007	0.9 ± 0.13
SLA	8.7 ± 7.39	29.1 ± 9.5
wt (129/B6)		
Medium	<0.007	0.05 ± 0.06
SLA	1.9 ± 2.0	2.5 ± 1.0
TNFRp75$^{-/-}$		
Medium	<0.007	0.09 ± 0.13
SLA	2.1 ± 1.8	5.1 ± 3.4
TNFRp55p75$^{-/-}$		
Medium	0.17 ± 0.15	0.52 ± 0.61
SLA	1.3 ± 1.0	9.5 ± 3.8

[a] Popliteal lymph node cells were cultured with medium or were stimulated with SLA for 72 h after harvesting cells. Quantiation of IFN-γ and IL-4 in the supernatants from 72-h cultures was performed by ELISA. IL-4 levels were equivalent to wt controls and were below 8 U/ml (data not shown). Data are representative of three similar experiments. Values represent the mean and SD for four to five mice. No significant differences ($p > 0.05$) were seen in IFN-γ production between TNFRp55$^{-/-}$ mice and wt (B6(7)) controls, nor were there significant differences between the wt (129/B6) and TNFRp75$^{-/-}$ or the TNFRp55p75$^{-/-}$ mice.

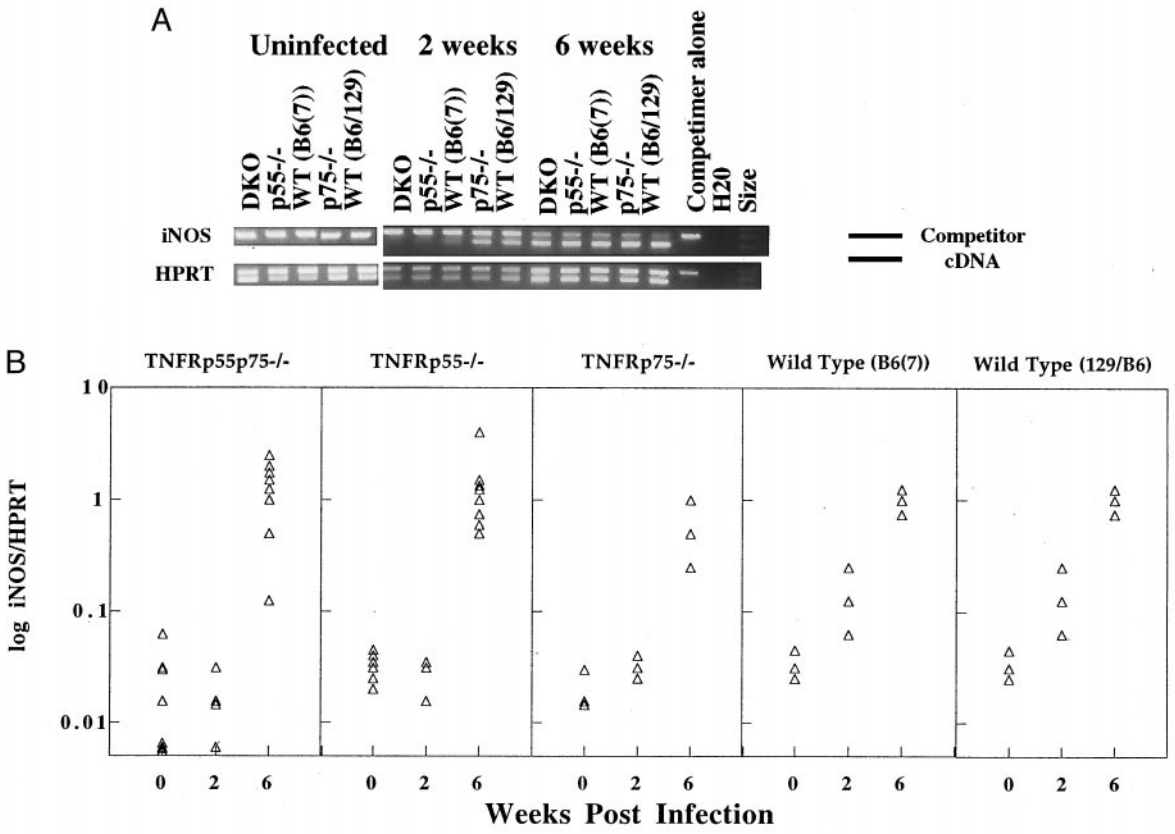

FIGURE 3. Expression of iNOS mRNA in *L. major* lesions from TNFR-deficient mice. RNA was harvested from lesions, and RT-PCR was performed. Samples were normalized to HPRT, and relative amounts of iNOS mRNA were determined by competitive PCR. *A,* Representative gel showing relative amounts of iNOS cDNA vs a constant amount (.025 pg/λ) of competimer. *Top band,* competimer. *Bottom band,* cDNA. Top row are products resulting from PCR with iNOS primers; bottom row are products using HPRT primers. *B,* iNOS/HPRT ratio from individual wt mice and TNFR-deficient mice. Increases over uninfected (time 0) compared with 2 wk postinfection were not significant ($p > 0.06$), whereas increases at 6 wk postinfection were significant ($p < 0.05$).

TNFRp75 $^{-/-}$ and TNFRp55p75$^{-/-}$ mice up-regulate iNOS mRNA at the site of infection by 6 wk postinfection

We previously demonstrated that mice lacking the TNFRp55 up-regulated iNOS mRNA in their lesions over time, correlating with earlier observations that resistant mice show an up-regulation of iNOS mRNA in their lesions compared with susceptible mice (16, 29). We were interested in determining what role the p75 receptor might play in iNOS mRNA expression. Therefore, we examined the expression of iNOS mRNA in the lesions of TNFR-deficient mice 2 and 6 wk postinfection. As seen in Figure 3*A*, iNOS mRNA is up-regulated in TNFRp55p75$^{-/-}$ and TNFRp75$^{-/-}$ mice by 6 wk postinfection. While we observed a slight increase in iNOS mRNA at 2 wk of infection in wt controls (Fig. 3*B*), this increase was not significant ($p > 0.06$). At 6 wk, we found significant increases ($p < 0.05$) in iNOS mRNA in all wt and TNFR-deficient mice compared with uninfected controls. Thus, it appears that iNOS gene expression can be induced in vivo in the absence of signaling through either TNF receptor.

Resident macrophages from TNFRp55$^{-/-}$ and TNFRp55p75$^{-/-}$ mice, but not TNFRp75$^{-/-}$ mice, exhibit a defect in macrophage activation

To determine whether macrophages lacking the TNFRp75 had any defect in activation, we infected resident macrophages in vitro with *L. major* amastigotes in the presence or absence of exogenous rIFN-γ. Resident macrophages from TNFRp75$^{-/-}$ and wt mice controlled the number of parasites per 100 macrophages with equal efficiency (Fig. 4, *A* and *D*). We also observed NO production by assessing the amount of NO_2 in the culture supernatant (22) in the presence of IFN-γ and amastigotes (Fig. 4*F*), consistent with previous observations that killing of parasites correlates with NO production in vitro (4, 30).

In contrast to macrophages from TNFRp75$^{-/-}$ mice, we found that resident macrophages from TNFRp55$^{-/-}$ mice were unable to control parasite replication or to produce NO, even when provided with high doses (200 U/ml) of IFN-γ (Fig. 4*C*). Similarly, resident macrophages from TNFRp55p75$^{-/-}$ mice also failed to control parasites or produce NO following IFN-γ activation (Fig. 4, *B* and *F*). Consistent with these findings were our results using neutralizing anti-TNF mAb (XT22.11) (Fig. 4, *E* and *F*). Thus, as previously shown (4), the ability of macrophages from wt mice (B6(7)) to become activated to produce NO and kill *L. major* was blocked when anti-TNF mAb was added. However, since thioglycollate-elicited macrophages from TNFRp55$^{-/-}$ mice can be activated with IFN-γ (16), and TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$ mice were able to produce NO and eliminate *L. major* in vivo, these results suggest that elicitation in vivo may be able to prime macrophages for NO production in a TNFRp55-independent manner.

In vivo activation of macrophages from TNFR-deficient mice

Because we observed a defect in macrophage activation in TNFRp55$^{-/-}$ and TNFRp55p75$^{-/-}$ macrophages in vitro, but were able to show increased iNOS gene expression and parasite elimination during infection, we hypothesized that during *L. major* infection other signals could prime macrophages for activation. To

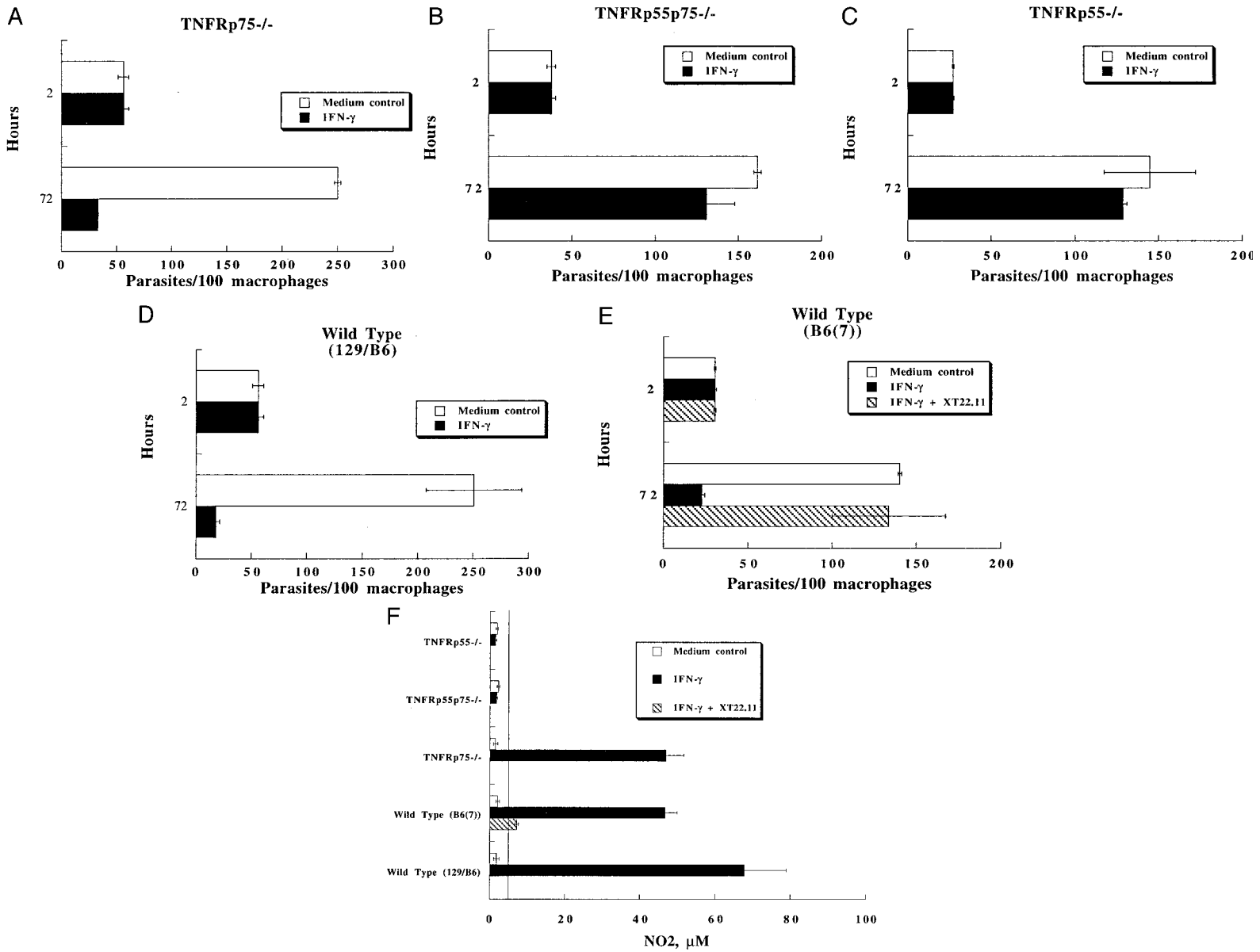

FIGURE 4. Activation of resident macrophages from TNFR-deficient mice. Resident macrophages were harvested from the peritoneal cavity and infected in vitro with amastigotes in the presence or absence of IFN-γ, and parasites and NO_2 were quantitated. *A-E*, The number of parasites per 100 macrophages is shown from macrophages from TNFRp75$^{-/-}$ (*A*), TNFRp55p75$^{-/-}$ (*B*), TNFRp55$^{-/-}$ (*C*), or wt (129/B6 (*D*) and B6(7) (*E*) incubated with or without 200 U/ml IFN-γ. *F*, NO (NO_2) in the culture supernatants of infected macrophages were quantitated by the Greiss reaction after 72 h. Limit of detection of the assay is 3.9 μM. Values shown are the mean ± SD from duplicate cultures. Results are representative of two experiments.

test whether macrophages could be primed in vivo for activation during the course of *L. major* infection, we harvested peritoneal macrophages from *L. major*-infected wt and TNFR knockout mice after elicitation with *Leishmania* parasites and assessed their ability to produce NO and kill *L. major* amastigotes. We found that without addition of exogenous rIFN-γ, macrophages elicited from uninfected mice of wt or knockout genotypes exhibited uncontrolled parasite replication and no NO production, (Fig. 5, open bars). However, macrophages harvested from 4-wk-infected wt mice produced NO in response to infection with amastigotes and controlled parasites over 72 h of culture (Fig. 5, solid bars). NO production was dependent upon a triggering stimulus, since incubating the elicited macrophages with medium alone failed to result in significant NO production (Fig. 5*B*). Thus, we were able to demonstrate killing by macrophages harvested from infected wt, TNFRp55$^{-/-}$, or TNFRp55p75$^{-/-}$ mice. Interestingly, macrophages elicited from uninfected mice were similar to resident macrophages, in that the absence of the TNFRp55 resulted in a defect in NO production and control of the parasites in the presence of recombinant IFN-γ over 72 h in culture (data not shown). Thus, the in vivo milieu provides compensatory signals for macrophage activation in the absence of the TNFRp55.

Discussion

In this study we found that mice control *L. major* infection in the absence of either the p55 or the p75 receptor, or both TNF receptors. Mice lacking only the TNFRp75 have a normal course of infection, while mice lacking both TNF receptors develop nonhealing lesions similar to those we previously reported in TNFRp55$^{-/-}$ mice (16). Our data show that TNF is not required for the development of Th1 cells or for activation of macrophages to eliminate *L. major*. The ability of mice to eliminate *L. major* without signaling through the TNF receptors suggests that TNF-α is not an absolute requirement for the development of protective immunity against this parasite and is in contrast to IFN-γ, IFN-γR, and IL-12p40, which are absolutely required for resistance (31–33).

IFN-γ-mediated macrophage activation is a critical part of the control of many intracellular pathogens, and TNF contributes to such activation. For example, maximal induction of NO in vitro requires treatment of macrophages with both TNF and IFN-γ (34). In leishmaniasis, it was shown that the ability of IFN-γ-primed macrophages to kill *L. major* amastigotes was dependent upon TNF, since addition of neutralizing anti-TNF mAb abrogated macrophage activation, results that we reconfirmed in this study (4). The required receptor for TNF signaling appears to be the p55 receptor, since resident macrophages from TNFRp55$^{-/-}$ or TNFRp55p75$^{-/-}$ mice were unable to produce NO or kill *L. major* in vitro, while macrophages from TNFRp75$^{-/-}$ mice were normal. Nevertheless, in contrast to these in vitro results, we found that mice were able to eliminate *L. major* in vivo. One explanation for these findings may be that macrophage activation is not responsible for parasite elimination in these animals. However, we were able to show that macrophages elicited from infected mice lacking TNF receptors were able to produce NO and control parasite replication. Thus, neither the p55 nor the p75 receptor is absolutely required for macrophage activation when priming occurs in vivo.

This result raises the obvious question as to how macrophages are activated in a TNF-independent manner during *L. major* infection. One possibility consistent with the data is that macrophage activation is dependent upon a T cell-macrophage interaction. Activated T cells, fixed activated T cells, and plasma membranes from activated T cells can synergise with IFN-γ to induce NO production by macrophages (35, 36). One candidate molecule that can transduce an activation signal is CD40, a member of the TNFR family. In fact, T cell activation of macrophages can be partially blocked with an antagonistic Ab to CD40L, and T cells from CD40L knockout mice are defective in their ability to activate macrophages (35). Interestingly, CD40- and CD40L-deficient mice exhibit enhanced susceptibility to *Leishmania*, although this enhanced susceptibility probably involves more than simply a lack of macrophage activation (37–39). Experiments are ongoing in our laboratory to investigate the role of CD40-CD40L interactions in TNFR-independent macrophage activation.

We previously reported that thioglycollate-elicited macrophages from TNFRp55$^{-/-}$ mice could be activated by high doses of IFN-γ (16) and postulated that the p75 receptor might compensate for the absence of the p55 receptor. Our results with macrophages harvested from TNFRp55p75$^{-/-}$ mice demonstrate that the p75 receptor is probably not essential in the absence of the p55 receptor and are similar to those reported by Yap et al. (11), who found that elicited macrophages from TNFRp55p75$^{-/-}$ mice also could be activated with high doses of IFN-γ.

It is useful to compare our findings with those reported following infection of TNFR-deficient mice with other intracellular pathogens. TNFRp55$^{-/-}$ mice infected with *M. tuberculosis* died by 20 days postinfection, while the wt controls survived (10). TNFRp55$^{-/-}$ mice failed to produce NO early after infection, although by 14 days iNOS levels were equivalent in the wt and the TNFRp55$^{-/-}$ mice. Similarly, infection of TNFRp55p75$^{-/-}$ mice with *Toxoplasma* was associated with up-regulation of iNOS gene expression, although, during the chronic phase of the disease, TNFRp55p75$^{-/-}$ mice died from severe toxoplasmic encephalitis (11). In the case of *Listeria*, infected TNFRp55$^{-/-}$ mice died within 5 days of infection (8, 9). Surprisingly, however, these mice had similar up-regulation of iNOS mRNA, compared with wt controls at 24 and 72 h postinfection (40). Reactive oxygen and nitrogen intermediates were found to be similar in the serum of TNFRp55$^{-/-}$ and wt controls, further supporting a TNFRp55-independent pathway for the regulation of these factors. While we found that neither TNF receptor is required for the eventual elimination of *L. major*, it appears that the TNF-independent pathway of parasite elimination is less efficient in controlling the infection. Thus, both TNFRp55$^{-/-}$ and TNFRp55p75$^{-/-}$ mice maintained a higher parasite burden than control animals at their peak of 6 wk postinfection and took considerably longer to eliminate parasites from the lesions. Macrophage activation contributes to the elimination of all these intracellular organisms, and the differences in the outcome of infection in TNFR-deficient mice with different pathogens might suggest that the delayed kinetics of NO production can be handled better in localized infections where pathology will not be life threatening (such as cutaneous lesions in leishmaniasis), than in systemic infections or those in which vital organs are affected. In some situations, however, severe disease in TNFRp55$^{-/-}$ mice may indicate that other TNF-dependent, iNOS-independent effector mechanisms that are required for eliminating particular pathogens are missing. This may be the case in *Listeria* (40).

We previously reported that, in spite of eliminating *L. major*, TNFRp55$^{-/-}$ mice are unable to resolve their lesions (16). We now show that TNFRp55p75$^{-/-}$ mice exhibit a similar phenotype, which eliminates the possibility that this unusual phenotype was associated with signaling via the p75 receptor. Similarly, it was recently reported that TNF-deficient mice are defective in controlling cell infiltration associated with *Corynebacterium parvum* infection (41). Signaling via the p55 receptor can lead to NF-κB activation or induction of apoptosis (7), and it is possible that TNF

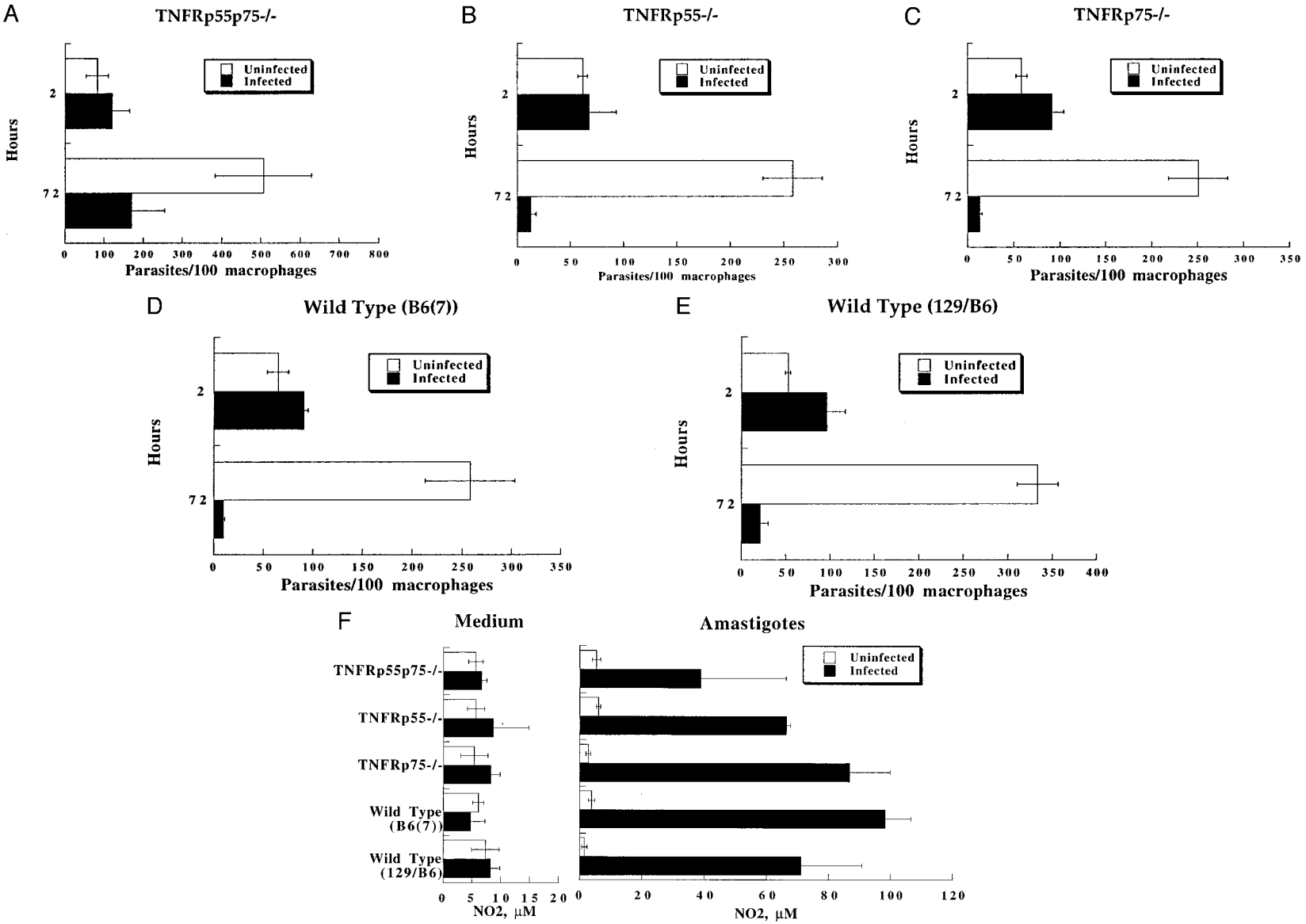

FIGURE 5. Activation of macrophages by elicitation of cells with Ag after infection. *A*, *L. major*-infected (4 wk) or uninfected mice were injected i.p. with freeze/thawed Ag. Ag-elicited macrophages were infected with amastigotes for 2 h, and aliquots were harvested at 2 and 72 h to determine the number of parasites per 100 macrophages. *A*, TNFRp55p75$^{-/-}$ mice. *B*, TNFRp55$^{-/-}$ mice. *C*, TNFRp75$^{-/-}$ mice. *D*, wt (B6(7)) mice. *E*, wt (129/B6) mice. $p < 0.05$ between infected and uninfected for all mice. *F*, NO (NO_2) production by Ag-elicited macrophages after 72 h in culture. $p < 0.05$ between infected and uninfected for all mice. *Left*, macrophages incubated in medium alone. *Right*, macrophages infected with amastigotes. C57BL/6 mice yield results identical to those of B6(7) mice. Data representative of three experiments. Values represent the mean ± SE of three individual mice.

is required for lymphocyte apoptosis and resolution of the inflammatory lesions associated with certain infections. TNF has also been shown to suppress T cell responses via modulation of TCR signaling, which could be another mechanism by which the absence of the p55 receptor could be associated with chronic inflammation (42). Studies are ongoing in our laboratory to investigate this issue.

The discovery that TNF binds to two receptors has generated a large amount of research to define the role that each receptor plays in mediating the effects of TNF, and the availability of genetically modified animals that lack one or both of these receptors has provided the tools to address this issue (8, 9, 14). Our work demonstrates that the p55 receptor plays a role in resident macrophage activation and lesion resolution. In contrast, the TNFRp75 is not required for either of these functions. Nevertheless, TNFRp55$^{-/-}$ and TNFRp55p75$^{-/-}$ mice were able to eliminate parasites following *L. major* infection, and parasite elimination was associated with NO production and macrophage activation. Future studies to elucidate how this TNF-independent pathway of macrophage activation functions will be important in fully understanding how intracellular pathogens are eliminated by the host's immune response.

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