A Critical Role for Interleukin 18 in Primary and Memory Effector Responses to *Listeria monocytogenes* that Extends Beyond its Effects on Interferon γ Production

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Abstract

The stimulation of interferon (IFN)-γ by interleukin (IL)-12 has been shown to provide protection from intracellular pathogens such as *Listeria monocytogenes*. Tumor necrosis factor (TNF) is also a major player in the resolution of *Listeria* infections and is suggested to have more global effects than can be explained by the induction of IFN-γ alone. Since IL-18 synergizes with IL-12 to induce IFN-γ production by natural killer and T helper (Th)1 cells, we determined its role in responses to *Listeria*. IL-18 appeared to be even more potent than either IL-12 or IFN-γ for protection against this pathogen and IL-18 enhanced bacterial clearance in the complete absence of IFN-γ. Indeed IL-18 was comparable to TNF in its ability to resolve the infection and showed a lowered protective capacity in the absence of TNF. Moreover, IL-18 induced macrophages to secrete both TNF and nitric oxide after a *Listeria* infection. IL-18 was also essential for optimal IFN-γ production by antigen-specific T cells. Therefore, IL-18 operates via its effects on both the innate immune response, including macrophages, as well as on Th1 cells, to protect against *Listeria*.

Key words: *Listeria* • IL-18 • TNF • NO • Th1 cells

Introduction

Listeria monocytogenes (*Listeria)*, a gram-positive facultative intracellular bacterium, is associated with severe infections in newborns, the elderly, and immunocompromised individuals (1–5). Murine models of listeriosis have been extensively studied to facilitate an understanding of the host immune response (6, 7). Results of these studies demonstrated that there is coordinate activation of the innate immune response, including neutrophils, macrophages, and NK cells (8, 9), together with the adaptive immune response of $CD4^+$ Th1 and $CD8^+$ T cells (6, 10). Moreover, it has been clearly demonstrated that for resolution and immunity to develop to *Listeria* the induction of antigen-specific T cells is essential (2, 10–12).

The release of proinflammatory cytokines from these various cell subsets was found to be pivotal in controlling primary immune responses to *Listeria*. IFN-γ, produced by NK and T cells, clears *Listeria* predominantly by enhancing the antimicrobial and antigen-presenting properties of macrophages (13–15). The key macrophage product, IL-12, provides protection from *Listeria* by inducing IFN-γ production (16). Likewise, TNF plays a role in the clearance of *Listeria* due in part to its induction of IFN-γ production (17, 18). However, IFN-γ does not completely replace the ability of TNF to resolve *Listeria* infections (16), in keeping with observations that TNF appears to be more dominant than IL-12 in eliciting protective primary immune responses to *Listeria* (16) yet plays a lesser role than IL-12 in invoking IFN-γ production by Th1 cells (19, 20). The role of these proinflammatory cytokines in the eradication of *Listeria* has also been confirmed in IFN-$\gamma^{-/-}$ (21), IFN-γ receptor$^{-/-}$ (22), IL-12$^{-/-}$ (23), and TNF receptor 1$^{-/-}$ (24, 25) mice.

In contrast to a primary immune response, the secondary immune response to *Listeria* is more rapidly induced and is able to overcome a high, normally lethal, dose of bacteria (6). This acquired resistance is largely attributable to memory effector $CD4^+$ and $CD8^+$ T cells, in part by IFN-γ

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production (26–28). Although IL-12 is also produced in a secondary challenge with this organism, it offers less protection than in primary responses and cannot completely account for the effects of IFN-γ (28), indicating that there may be other players involved in IFN-γ production at this stage. TNF is clearly protective in secondary responses to *Listeria*, and this cytokine may complement the role of IFN-γ in the bacterial clearance (27, 29). In addition to the role of $CD4^+$ and $CD8^+$ T cells in the memory response to *Listeria*, the innate immune response is also essential with neutrophils playing a critical role (30, 31).

A potentially important player in the eradication of a *Listeria* infection is IL-18 (32), which was originally shown to induce IFN-γ production in mice injected with heat-killed *Propionibacterium acnes* (*P. acnes*) and LPS (32, 33). In vitro studies have since shown that IL-18 synergizes with IL-12 to induce IFN-γ from differentiating and committed $CD4^+$ Th1, but not Th2, cells (34) as well as from NK cells (35). These two cytokines can also synergize in vivo since IL-$12^{-/-}$IL-$18^{-/-}$ mice displayed more profound impairment of IFN-γ production after injection with *Mycobacterium bovis* than did either IL-$12^{-/-}$ or IL-$18^{-/-}$ mice individually (36). In addition to its IFN-γ–enhancing capacity, IL-18 can also augment the cytotoxic activity of both NK and T cells (36–40) and can enhance their production of other proinflammatory mediators (33, 36, 41–43). Moreover, studies of acute bacterial, fungal, parasitic, and viral infections have shown a role for IL-18 in the eradication of intracellular pathogens (44–49). However, a role for IL-18 in memory effector responses to such pathogens has not been investigated yet, nor has its ability to protect through mediators other than IFN-γ.

Responsiveness to IL-18 is conferred by binding to its cognate receptor, which consists of an IL-1R5/IL-1R7 heterodimer (50–52). Herein, using a neutralizing mAb to the IL-1R7 chain (anti–IL-18R; reference 53) we report an important and partly IFN-γ–independent role for IL-18 in both innate and adaptive immune responses to *Listeria* involving the production of TNF and nitric oxide (NO) as well as IFN-γ.

Materials and Methods

Mice. Female Balb/c and C.B-17 severe combined immunodeficient (SCID) mice (both H-2d) were purchased from Taconic Farms. Female Balb/c IFN-γ–deficient mice (H-2^d) were provided by Bob Coffman (DNAX Research Institute, Palo Alto, CA). Female mice transgenic for the DO11.10 αβ TCR (54) on a Balb/c genetic background were identified at age 4–6 wk by staining peripheral blood leukocytes with the anti-TCR clonotype-specific mAb KJ1-26 (55); these mice were heterozygous for the TCR-α and -β transgenes. All mice were housed under specific pathogen-free conditions and were used at the age of 6–8 wk.

Bacteria and Bacterial Antigens. *Listeria* (provided by H. Rogers, K. Murphy, and E. Unanue) has been maintained in a virulent state by repeated passage in mice. Bacteria were grown in BHI broth (Difco) to midlog phase as determined by OD_{560} measurements and aliquots were stored in 20% glycerol/PBS at –80°C.

Heat-killed *Listeria monocytogenes* (HKLM)* was obtained by incubating live bacteria at 74°C for 120 min. Aliquots were stored at −80°C.

mAbs, Cytokines, and Reagents. The mAbs anti-β galactosidase (isotype control: GL117), anti–IL-12 (C17.8.20), anti–IL-18R (TC30-28E3), and anti-TNF (XT22) were administered (1 mg) intraperitoneally to mice 1 h before infection with *Listeria* and at the additional times as indicated. Recombinant mouse cytokines were IL-2 (DNAX), TNF (Genzyme), IL-12 (BD PharMingen), and IL-18 (PeproTech). LPS was obtained from Sigma-Aldrich and azide-free, low endotoxin anti-CD3ε was obtained from BD PharMingen. The antigenic peptide from chicken OVA ($OVA_{323–339}$) was synthesized on an Applied Biosystems model 430 peptide synthesizer.

Infection with Listeria. An initial infection with *Listeria* was performed by intravenous injection of 1–2 × 10^3 CFU viable bacteria diluted in a volume of 0.2 ml PBS. For a second infection, mice were injected with 5 × 10^4 CFU viable bacteria on day 28 after the initial infection. Actual numbers of bacteria injected were determined by plating aliquots of relevant dilutions on BHI agar (Difco). Bacterial growth in the spleen was determined on the indicated days after infection by plating relevant dilutions of the spleen homogenates on BHI agar. Colonies were counted after 24 h of incubation at 37°C. LD50 were calculated as described previously (56).

Cell Culture Ex Vivo. On the indicated day after infection, single cell spleen suspensions were prepared from individual mice in culture medium: RPMI 1640 medium (Bio-Whittaker) supplemented with 10% FCS (UT; Hyclone Laboratories), 1 mM L-glutamine, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 0.05 mM β-2 mercaptoethanol (all GIBCO BRL). Cells were incubated in 96-well plates at a density of 10^6 cells per well with 3.9 × 10^4 CFU HKLM for 48 h at 37°C. Supernatants were harvested at the end of culture and stored at −80°C until analyzed for cytokine content.

T Cell Culture In Vitro. $CD4^+$ T cells were enriched from DO11.10 spleen cell preparations by negative selection using magnetic-activated cell sorting with a mixture of biotinylated anti-CD8α, anti–I-A^d, anti-B220, anti-GR1, and anti–Mac-1 mAbs (Miltenyi Biotec). The enriched cells were then purified further using a FACStarPlus™ flow cytometer (Becton Dickinson) to achieve >99% $CD4^+$ T cells, demonstrated to be naive on the basis of bright L-selectin staining (57).

Primary stimulations of these sorted $CD4^+$ T cells (2.5 × 10^5 cells per well) were conducted using 0.6 μM $OVA_{323–339}$, 3.9 × 10^4 CFU HKLM, and RBC-lysed Balb/c spleen cells (5 × 10^6 cells per well; 3,000 rad) as APCs with or without the addition of 10 μg/ml of the anticytokine mAbs described above (all at 10 μg/ml). T cells were expanded threefold into fresh culture medium at 72 h and cells harvested on day 7 were washed three times, counted, and restimulated with fresh APCs, OVA plus or minus HKLM with or without the addition of anticytokine mAbs as detailed in the individual figure legends. All concentrations used for restimulation were the same as those described for priming. Supernatants were collected at 48 h and stored at −80°C until analyzed for cytokine content.

Macrophage Cultures In Vitro. Macrophages from peritoneal exudates were selected by retaining adherent cells. After overnight culture in medium at 2 × 10^6 cells per milliliter in flat-bottomed 96-well plates, adherent or whole peritoneal cell populations were placed in the presence of IL-18 (100 ng/ml) or

Abbreviation used in this paper: HKLM, heat-killed *Listeria monocytogenes*.

HKLM (3.9×10^4 CFUs) with or without anti–IL-18R (10 μg/ml) for 48 h, and supernatants were stored at −20°C until assayed for cytokine content. For intracellular cytokine staining, identical cultures were stimulated in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich) for 4 h.

Alternatively, whole peritoneal cell populations were incubated at 10^5 cells per milliliter in flat-bottomed 96-well plates with HKLM (3.9×10^4 CFUs) for 16 h and 50 μl of supernatant measured for nitrite content using 50 μl Greiss reagent (3% phosphoric acid, 1% *p*-amino-benzene-sulfonamide, and 1% *n*-1-napthylethylenediamide; Sigma-Aldrich) (58) and absorbance was read at 540 nm.

Cytokine Assays. IFN-γ was detected using a two-site sandwich ELISA as described previously (59). The sensitivity was 125 pg/ml (1 U/ml = 0.1 ng/ml). TNF was detected using a Quantikine™ M ELISA (R&D Systems) according to the manufacturer's instructions. The sensitivity was <5 pg/ml. TNF-secreting cells were analyzed on a single cell basis by flow cytometric analysis of intracellular TNF as described previously (60).

Histology and Immunohistochemistry. Tissue for immunohistochemistry was immersed in OCT compound (TissueTek) and frozen in the vapor phase of liquid N_2. 8-μm frozen sections were thawed on gelatinized microscope slides, allowed to dry at room temperature, and fixed in acetone containing 0.04% (vol/vol) H_2O_2 (room temperature, 10 min). Primary and secondary mAbs were diluted in PBS containing 7.5% (wt/vol) BSA, 10% (vol/vol) normal mouse serum, and 10 mM NaN_3. Primary mAbs were purified RM4-5 (anti–mouse CD4), purified 53–6.7 (anti–mouse CD8), purified or FITC-conjugated RB6-8C5 (anti-Ly6G, GR-1), purified or FITC-conjugated F4/80 (mouse macrophages), and the appropriate isotype controls (all BD PharMingen). Secondary mAbs were peroxidase-conjugated donkey-anti–rat (Jackson ImmunoResearch Laboratories) or peroxidase-conjugated sheep-antifluorescein (Roche). All mAbs were used at previously determined concentrations (10–50 μg/ml). Peroxidase activity was visualized using diaminobenzidine (DAB; Sigma-Aldrich). Section were lightly counterstained with hematoxylin solution (Sigma-Aldrich), dehydrated, and mounted in Hemode (Fisher Scientific).

Statistical Analysis. The Dunnet procedure for all possible pairwise contrasts of means with a control mean was performed as indicated.

Results

IL-18 Is Critical for Resistance to Listeria. To obtain quantitative information on the effect of IL-18 on survival to *Listeria,* mice were infected with graded doses of the organism in the presence of a neutralizing mAb to IL-18R (53). This was compared with an isotype control or anti–IL-12 mAb, since IL-12 is known to protect from *Listeria* (61). For those mice treated with an isotype control mAb (Fig. 1) or PBS (data not shown), the highest dose of 5×10^4 CFUs led to 100% mortality within 4 d, whereas 10^3 CFUs was sublethal for 100% of these animals. Administration of anti–IL-18R dramatically increased the susceptibility to *Listeria* with 100% of mice now succumbing to as little as 10^3 CFUs by day 4 (Fig. 1 and Table I). In contrast, anti–IL-12 had no effect on the survival of mice infected with 10^3 CFUs (Fig. 1). In fact, 100% mortality was not observed in anti–IL-12–treated mice until 5 d after they were infected with ≥10^4 CFUs (Fig. 1 and Table I). This dose was lethal for <50% of isotype control mice (Fig. 1) and their mean survival was extended to 6 d (Table I) suggesting that IL-12 was playing a protective role in the infection albeit to a lesser extent than IL-18. Impaired survival was seen to a similar extent whether mice were treated with anti–IL-18R alone or in conjunction with anti–IL-12 (Fig. 1 and Table I). Hence these findings indicate that IL-18 may play a more dominant role than IL-12 in survival from a *Listeria* infection.

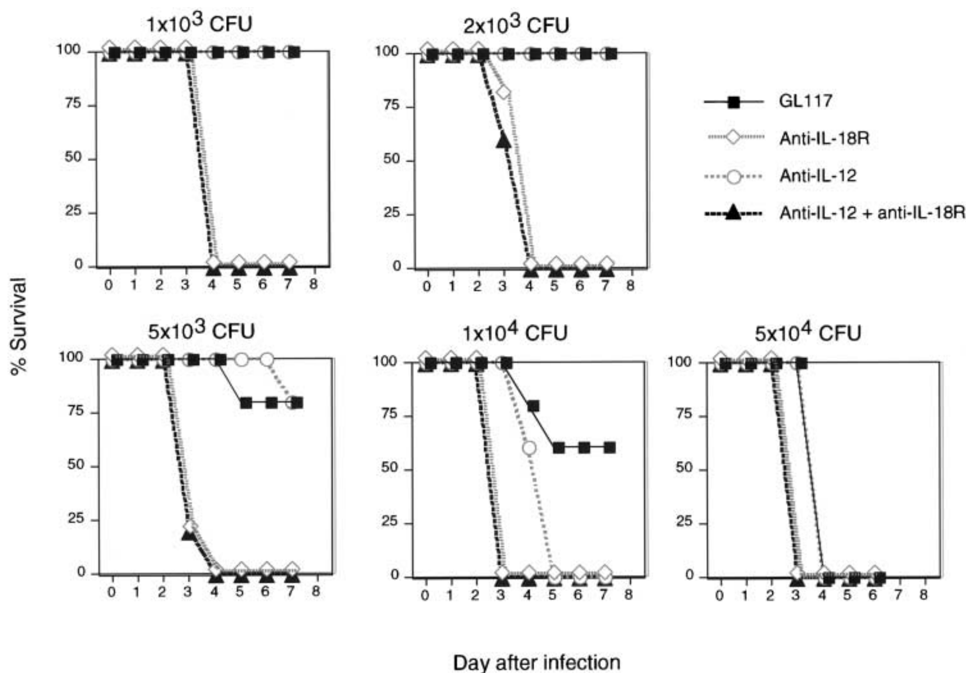

Figure 1. Anti–IL-18R treatment can increase sensitivity to *Listeria* to a greater extent than anti–IL-12. Balb/c mice were injected weekly with isotype control mAb (GL117), anti–IL-12, anti–IL-18R, or the combination of both anticytokine mAbs before infection with the indicated doses of *Listeria* as described in Materials and Methods. Survival was monitored over the next 8 d and plotted as a percentage of the total number of mice per group. 6 mice per group were assessed and the results shown were representative of three experiments.

Table I. *Mean Survival Time*

Dose of *Listeria* (CFUs):	10^3	2×10^3	5×10^3	10^4	5×10^4
Group					
GL117	ND	ND	ND	6	4
Anti–IL-18R	4	4	3	3	3
Anti–IL-12	ND	ND	ND	5	4
Anti–IL-12 plus anti–IL-18R	4	4	3	3	3

Displayed is the mean survival time (in days) of mice from the experiment described in Fig. 1.

IL-18–mediated Protection to Listeria Occurs even in the Absence of IFN-γ. Since IL-18 synergizes with IL-12 for IFN-γ production by NK and Th1 cells (34, 36) and the listericidal effects of IL-12 have been shown to be IFN-γ dependent (61), we determined how IL-18 compared with IL-12 and IFN-γ in its ability to resolve a *Listeria* infection. Clearance of *Listeria* from the spleen 3 d after infection was significantly impaired in those animals pretreated with anti–IL-18R mAb as compared with isotype control mAb, whether or not *Listeria*-infected mice were fully immunocompetent (Balb/c mice, Fig. 2 A) or lacked T and B cells (CB17.SCID mice, data not shown). This correlated with a significant reduction in the levels of IFN-γ produced upon antigen stimulation of spleen cells from the anti–IL-18R mAb treated group versus the isotype mAb treated controls (Fig. 2 B). Bacterial clearance was also reduced in *Listeria*-infected mice given anti–IL-12 or anti–IFN-γ mAbs as compared with isotype mAb treated control mice (Fig. 2 A) and as reported previously (16). However, the bacterial burden remained significantly lower in the anti–IL-12 or anti–IFN-γ mAb treated mice than in the anti–IL-18R mAb treated mice even though the antigen-specific IFN-γ levels of each group were impaired similarly (Fig. 2 B). There was no additive effect between anti–IL-18R and anti–IL-12 since the CFU counts when both mAbs were given together did not differ from those obtained using the individual mAbs (Fig. 2 A). Taken together, our observations reveal a critical role for IL-18 in the clearance of *Listeria,* which cannot be fully explained by its effects on IFN-γ production.

To rule out the possibility that our findings that IL-18 was more dominant than IFN-γ in bacterial clearance was due to differing mAb efficacies, we also analyzed Balb/c.IFN-γ–deficient mice which are more susceptible to *Listeria* than regular Balb/c mice (Fig. 2 C; reference 21). Bacterial clearance was again increased significantly beyond isotype control levels by administering anti–IL-18R before infecting these mice (Fig. 2 C).

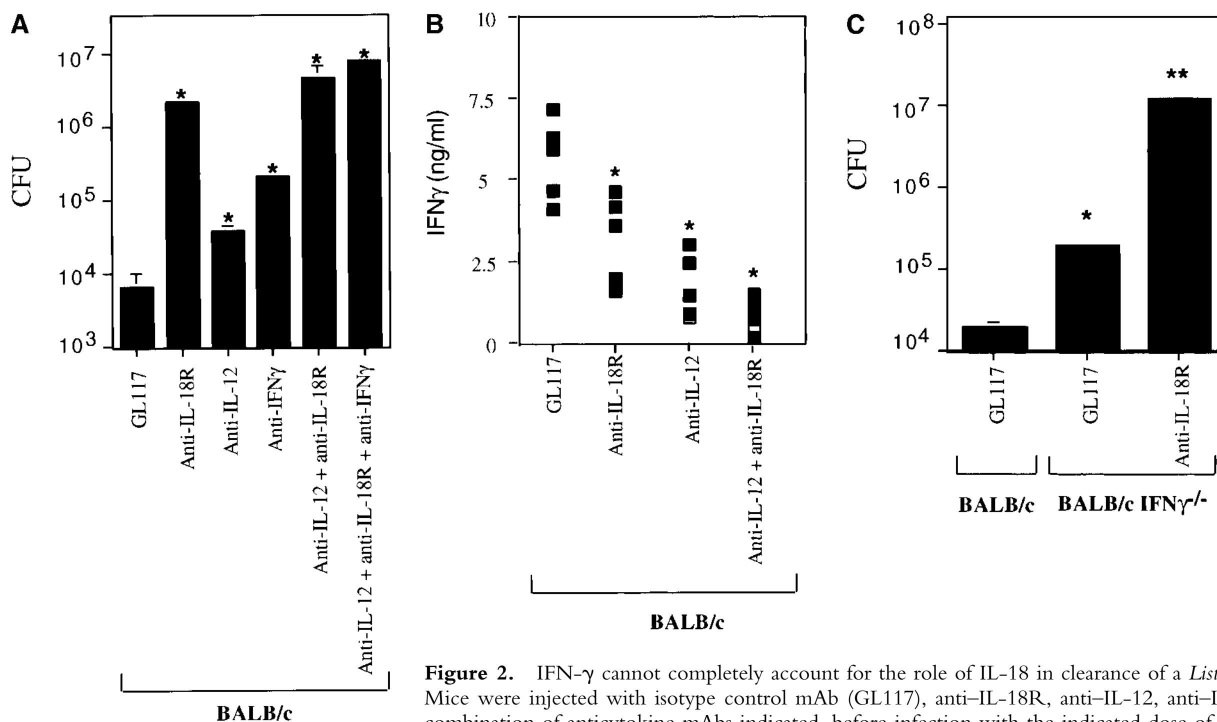

Figure 2. IFN-γ cannot completely account for the role of IL-18 in clearance of a *Listeria* infection. Mice were injected with isotype control mAb (GL117), anti–IL-18R, anti–IL-12, anti–IFN-γ, or the combination of anticytokine mAbs indicated, before infection with the indicated dose of *Listeria* as described in Materials and Methods. (A) The bacterial burden per spleen of Balb/c mice was evaluated 3 d after infecting with 2×10^3 CFUs. Each bar represents the mean CFU per group ± SD for ≥5 mice per group. (B) Spleen cells from the same Balb/c mice were also restimulated ex vivo with 3.9×10^4 CFU HKLM for 48 h and supernatants analyzed for IFN-γ. Data are shown for individual mice. (C) The bacterial burden per spleen of Balb/c and Balb/c.IFN-$\gamma^{-/-}$ mice was evaluated 3 d after infecting with 600 CFUs. Each bar represents the mean CFU per group ± SD for ≥5 mice per group. Results are representative of greater than three experiments. Statistical analysis was performed using Dunnett's. *$P < 0.05$ versus Balb/c isotype control. **$P < 0.05$ versus Balb/c.IFN-$\gamma^{-/-}$ isotype control.

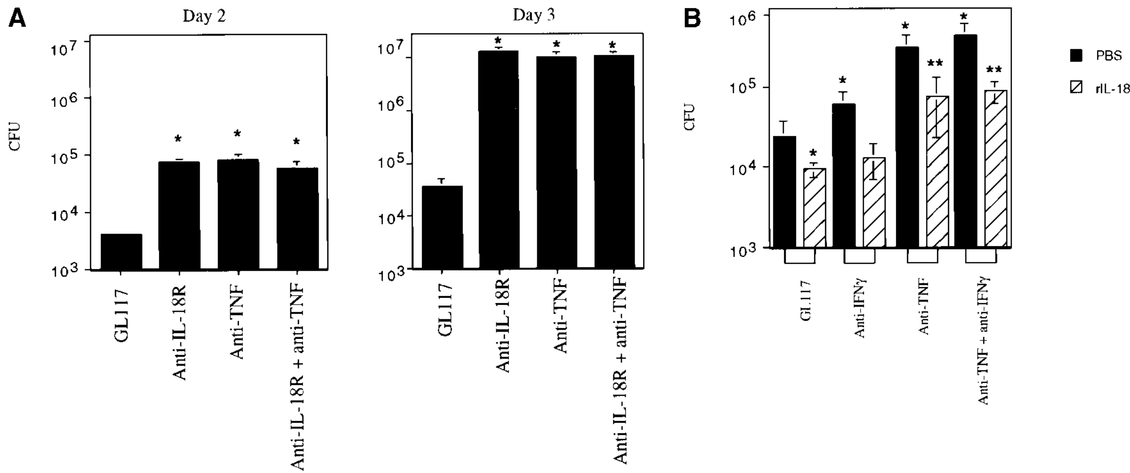

Figure 3. TNF contributes to the protective effects of IL-18. (A) Balb/c mice were injected with the mAbs indicated before infection with 2×10^3 CFU *Listeria* and the bacterial burden per spleen was evaluated after 2 or 3 d. (B) Balb/c mice treated with the mAbs indicated were given 2×10^3 CFU *Listeria* and treated daily with either 0.3 ml PBS or an equal volume containing 5 μg rIL-18 as indicated. The bacterial burden per spleen was evaluated after 2 d. Each bar represents the mean CFU per group ± SD for ≥5 mice per group. Results were reproducible in greater than three experiments. Statistical analysis was performed using Dunnett's. $^*P < 0.05$ versus isotype control plus PBS. $^{**}P < 0.05$ versus isotype control plus rIL-18.

TNF Contributes to the Protective Effects of IL-18. Since IL-18 can induce TNF in nonpathogen-driven systems (62–64) and TNF is critical in eradicating *Listeria* (17, 18), we determined how these two cytokines compared in their ability to clear *Listeria*. Strikingly, anti–IL-18R had a similar capacity to anti-TNF in impairing bacterial clearance (Fig. 3 A). Those mice given either anti–IL-18R or anti-TNF mAb harbored colonies of *Listeria* in their spleen 2.5 logs higher than isotype mAb treated control mice by the third day of infection (Fig. 3 A). In addition, there was no additive effect between anti–IL-18R and anti-TNF since the CFU counts when both mAbs were given together did not differ from those obtained using the individual mAbs when assessed 2 d after infection and therefore, before the CFU plateau was reached (Fig. 3 A). Thus, we assessed bacterial clearance at this earlier time of infection in mice given mAbs to TNF and/or IFN-γ with or without a daily treatment of rIL-18 to determine whether or not IL-18 required TNF and/or IFN-γ for its protective effects.

The bacterial burden of isotype mAb treated control mice was indeed significantly reduced by administering rIL-18 (Fig. 3 B). When rIL-18 was given to mice depleted of IFN-γ the bacterial load was reduced to the level seen with the isotype controls given rIL-18 (Fig. 3 B) confirming that this treatment could prevent an otherwise exacerbated infection. In contrast, although rIL-18 treatment reduced the bacterial burden of mice given mAbs to TNF to a significant extent, it did not reduce it to the level seen with either the rIL-18–treated isotype controls or anti–IFN-γ–treated mice given rIL-18 (Fig. 3 B). The effects of rIL-18 on bacterial clearance were similar when mice had been treated with anti-TNF mAbs alone or in combination

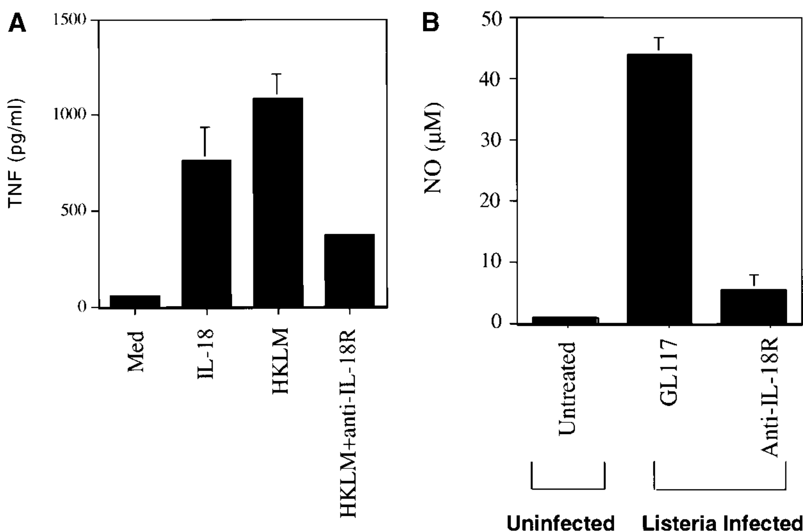

Figure 4. IL-18 induces TNF and NO production by macrophages from *Listeria*-infected mice. (A) Adherent peritoneal cells taken from SCID mice 3 d after infecting with 2×10^3 CFU *Listeria* were rested overnight and stimulated for 48 h with medium, IL-18, HKLM, or HKLM plus anti–IL-18R at concentrations detailed in Materials and Methods. The supernatants were assessed for TNF content by ELISA and data are represented as the mean ± SD of triplicate cultures. (B) Adherent peritoneal cells from SCID mice left untreated or taken 3 d after treating with the mAbs indicated ± infecting with 2×10^3 CFUs *Listeria* were stimulated for 16 h with HKLM and supernatants assessed for nitrite content as described in Materials and Methods. Data are represented as the mean ± SD of duplicate cultures.

with anti–IFN-γ mAbs. Overall these findings indicate that the protective capacity of IL-18 largely required the production of TNF and did not appear to require IFN-γ in this early phase of bacterial clearance.

IL-18 Is Required for TNF and NO Production by Listeria-infected Macrophages. *Listeria*-infected mice given anti–IL-18R were not impaired in their antigen-specific production of TNF upon restimulation with HKLM in vitro (data not shown). Therefore, we investigated the macrophage as an alternative source of TNF in response to IL-18, using adherent cells from the peritoneal cavity of *Listeria*-infected SCID mice. In these cultures the release of TNF was completely restricted to F4/80$^+$ macrophages (data not shown) and was increased by stimulation with either IL-18 or HKLM (Fig. 4 A). Moreover, the TNF produced in response to HKLM was significantly reduced upon addition of an anti–IL-18R mAb (Fig. 4 A). Overall these findings show that IL-18 enhanced the production of TNF by macrophages from *Listeria*-infected mice. However, no effect on macrophage TNF regulation ex vivo was observed after *Listeria* infection in the presence of anti–IL-18R as compared with isotype control in vivo (data not shown) since the macrophage produces IL-18 upon stimulation in vitro thus masking the effects of anti–IL-18R treatment in vivo.

NO forms part of a critical effector pathway involved in eliminating bacteria from the macrophage (65–67). We tested if this factor was also influenced by IL-18 using peritoneal (or splenic, data not shown) macrophages from SCID mice. In comparison to cells taken from noninfected mice, cells from *Listeria*-infected mice produced far higher levels of NO upon restimulation with HKLM (Fig. 4 B). Anti–IL-18R treatment before infection greatly impaired this response (Fig. 4 B) indicating that IL-18 was also required for this macrophage effector function. In addition, NO levels from all groups were lowered by addition of anti–IL-18R to the cultures themselves (data not shown).

Inhibition of IL-18 Action in Listeria Infection Greatly Enhances Neutrophilic Microfoci. Livers from *Listeria*-infected mice treated with anti–IL-18R showed a dramatically increased GR1 staining for neutrophils, as compared with controls and this was restricted to microfoci within the tissue (Fig. 5). The frequency of neutrophil-rich microfoci in mice treated with mAbs to either IL-18R or TNF was similar and greater than seen using either anti–IFN-γ or anti–

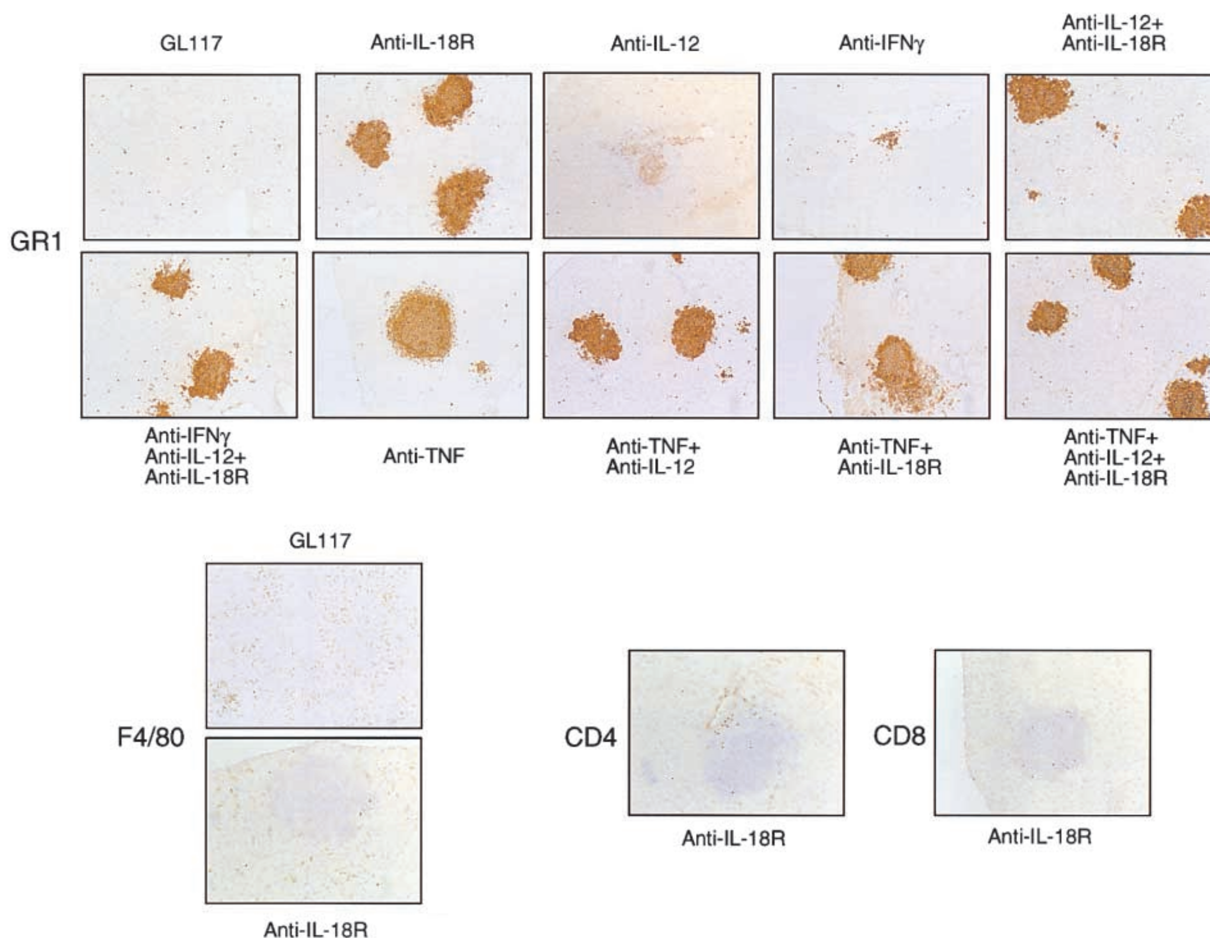

Figure 5. Histological analyses of *Listeria*-infected mouse livers confirms the dominant role of IL-18 and TNF in primary immune responses to *Listeria* infection. Displayed are frozen sections from the livers of *Listeria*-infected Balb/c mice treated with the mAb combinations indicated and stained with either GR1, F4/80, CD4, CD8, or isotype control (data not shown) mAbs as described in Materials and Methods.

IL-12 mAbs, while the liver appeared normal by day 3 after infection in control animals (Fig. 5). Hence the frequency of these microfoci in the liver correlated completely with the capacity of each mAb to impair bacterial clearance in the spleen (Fig. 2 A and Fig. 3), but not with IFN-γ production (Fig. 2 B). F4/80 expression was only found on Kupffer cells distributed throughout the liver parenchyma, but not in the microfoci themselves, and was similar in all groups (Fig. 5), suggesting that additional macrophages had yet to influx the site at this time point. No CD4 and CD8 staining was found in the control group and only a few $CD4^+$ or $CD8^+$ cells were seen at the edge of the microfoci of those groups with pathology (Fig. 5).

IL-18 Has a Greater Effect on Th1 Effector Cells after their Differentiation. Our studies suggest that IL-18 induced clearance of *Listeria* in part through its ability to increase IFN-γ production, which is in keeping with its reported ability to synergize in vitro with IL-12 for the induction of IFN-γ by NK and Th1 cells (34, 68). We assessed the effect of anti–IL-18R on the development of Th1 cells in vitro by stimulating $CD4^+$ Mel-14^{hi} cells from DO11.10 mice with HKLM, $OVA_{323-339}$, and APCs for 1 wk in the presence or absence of mAbs to IL-18R, IL-12, or TNF. The generation of Th1 cells was analyzed by measurement of IFN-γ production after restimulating with OVA and APCs. Results are shown in the absence of HKLM but were identical whether or not HKLM was present in these secondary recall cultures. IL-12 has been previously shown to play a dominant role in Th1 cell development in contrast to TNF (19, 20). We now show that anti–IL-18R only slightly reduced the optimal development of Th1 cells seen in the absence of any blocking mAbs (Fig. 6 A). However, as suggested from our earlier studies (34), anti–IL-18R had a more pronounced effect on IFN-γ production during the recall responses of Th1 cells already developed in the absence of blocking mAbs for 7 d with HKLM, OVA, and APCs (Fig. 6 B). However, this was still less effective than anti–IL-12, which had a more prominent effect at this recall stage (Fig. 6 B). Anti-TNF had minimal effects not only in modulating the levels of IFN-γ detected from primary development but also in recall cultures (Fig. 6, A and B) as shown previously (20, 69). The role of IL-18 in enhancing IFN-γ production from already differentiated Th1 cells suggested that this cytokine might be critical for the optimal maintenance of Th1 responses necessary to generate potent effector memory cells, which are required for effective bacterial clearance of repeated *Listeria* infections.

IL-18 Is a Dominant Factor in the Memory Effector Response Induced upon Reinfection with Listeria. To test the role of IL-18 in memory effector responses to *Listeria*, mice given an initial sublethal *Listeria* infection of 2×10^3 CFUs were administered with anticytokine mAbs 27 d later, and then reinfected with a high dose of 5×10^4 CFU *Listeria* on day 28. The animals were assessed for bacterial burden and IFN-γ production after a further 3 d. Isotype-treated control mice were now able to completely clear this bacterial infection which is otherwise lethal in a primary response (Fig. 7 A) and this correlated with high levels of IFN-γ

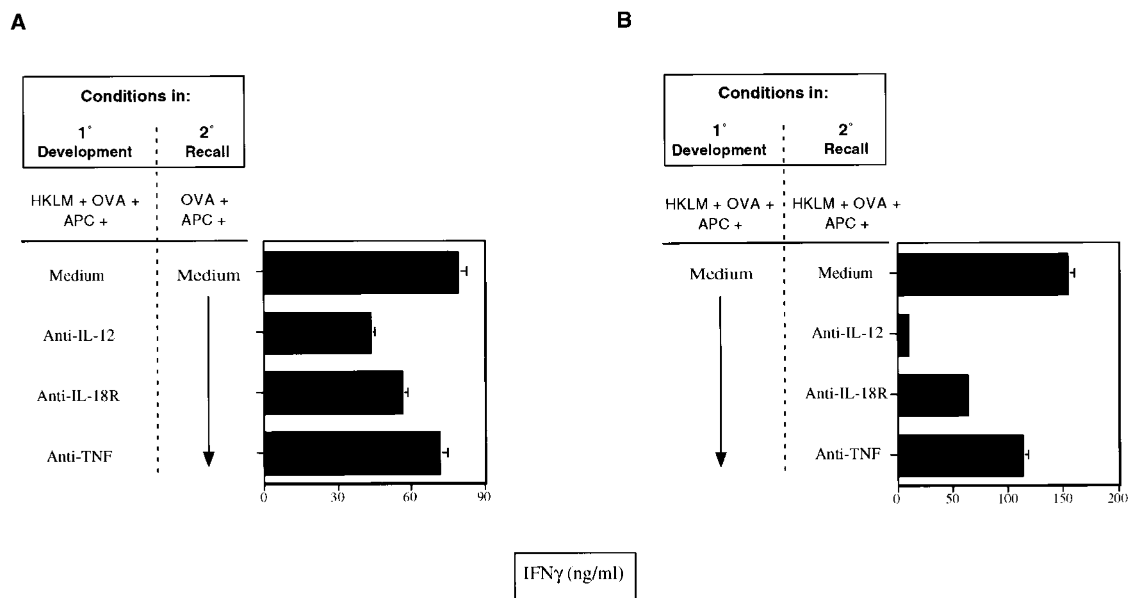

Figure 6. IL-18 has a greater effect on Th1 cells after their differentiation in vitro. Naive $CD4^+$ spleen cells sorted from DO11.10 transgenic mice were set up in primary cultures with HKLM, OVA, and APCs (irradiated spleen cells) for 7 d. (A) Primary T cell cultures were performed in the presence of medium or mAbs to IL-12, IL-18R, or TNF as described in Materials and Methods. Cells were then restimulated with OVA plus APCs for 48 h and supernatants harvested to determine IFN-γ content. (B) Primary T cell cultures were stimulated with HKLM, OVA, and APCs in the absence of mAbs for 7 d and then restimulated with HKLM plus OVA plus APCs for 48 h in the presence of medium or mAbs to IL-12, IL-18R, or TNF when supernatants were harvested to determine IFN-γ content. All possible combinations of Ab were also analyzed in this experiment and their effects on lowering IFN-γ production were found to be additive (results not shown). Results were representative of three experiments and data are represented as the mean ± SD of triplicate cultures.

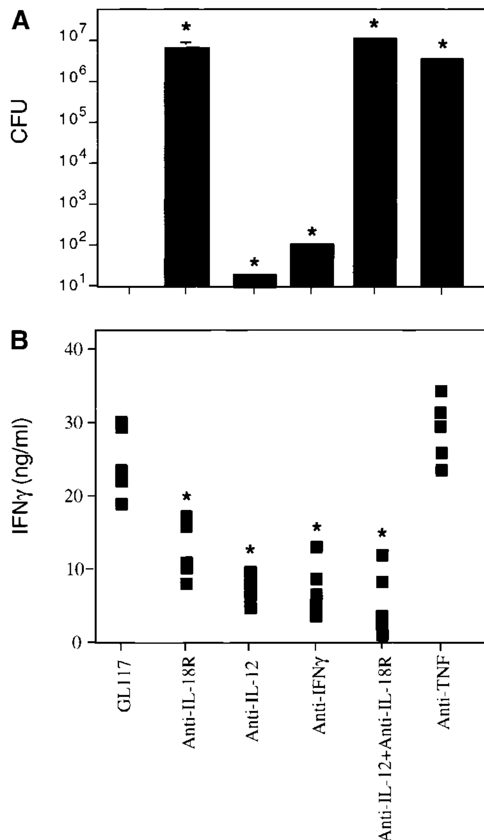

Figure 7. A critical role for IL-18 in memory recall responses and subsequent clearance of a repeat *Listeria* infection. Balb/c mice were infected with 2×10^3 CFU *Listeria* and 27 d later groups were treated with the indicated mAbs and then reinfected with 5×10^4 CFU *Listeria* on day 28. After a further 3 d the bacterial burden per spleen (A) and IFN-γ production by spleen cells upon restimulation with HKLM antigen for 48 h (B) were evaluated as described for Fig. 2. Results were representative of three experiments. Statistical analysis was performed using Dunnett's. $^*P < 0.05$ versus isotype control.

production upon antigenic stimulation (Fig. 7 B). In contrast to the controls, treatment with anti–IL-18R mAb led to a greatly enhanced bacterial burden, which was significantly greater than that observed after treatment with either anti–IL-12 or anti–IFN-γ mAbs (Fig. 7 A). This profound role of IL-18 in bacterial clearance was not completely attributable to reduction in the levels of IFN-γ. Specifically, treatment with anti–IL-18R mAb reduced the levels of IFN-γ to the same extent yet exacerbated the bacterial load significantly more than either anti–IL-12 or anti–IFN-γ mAbs (Fig. 7, A and B). Indeed, the dominance of anti–IL-18R over anti–IL-12 was even more dramatic in impairing recall responses than had been observed in primary responses to *Listeria*. The effect of anti–IL-18R mAb on bacterial clearance was comparable to that of anti-TNF, although anti-TNF showed no effect on antigen-induced IFN-γ production (Fig. 7). At this challenge dose, there was no further enhancement in bacterial load using anti–IL-18R in combination with Abs to TNF, IFN-γ, or IL-12 (Fig. 7 A, and data not shown).

In keeping with the similar effects of anti–IL-18R mAb in primary and secondary *Listeria* infections, the livers of mice treated with this mAb before a secondary infection also contained very high numbers of GR-1$^+$ neutrophils within microfoci (data not shown). This is in keeping with an important role for IL-18 in the induction of recall responses.

Discussion

This study was performed to determine the role of IL-18 in protective immune responses to the intracellular pathogen *Listeria*. Our findings show that IL-18 provided significant protection in both primary and secondary responses to this pathogen and appeared more potent than either IL-12 or IFN-γ. Thus, IL-18 has a more extensive role in bacterial clearance than via its ability to induce IFN-γ production by Th1 and NK cells, since it can exacerbate the bacterial load even in IFN-γ–deficient mice. The main effects of IL-18 were observed only in the presence of TNF. Indeed, IL-18 induced macrophage production of TNF as well as NO, which is critical for its antimicrobial effects. Overall, our findings show that IL-18 plays a very important role in cell-mediated immunity via its effects on both the innate and adaptive immune response, which appear important for both primary and secondary challenge with this intracellular pathogen. Thus, IL-18 may prove to be beneficial when used as part of an antimicrobial treatment or when targeted as a therapeutic strategy for those disorders associated with Th1 inflammatory responses.

Our data show that IL-18 plays a dominant role in the clearance of *Listeria* in a manner not fully attributable to the induction of IFN-γ. Evidence for this came from the following findings. First, the bacterial load and mortality was higher in the absence of IL-18 than in the absence of IL-12, while the production of IFN-γ was reduced similarly in each case. Secondly, IL-18, but not IL-12, was dominant over IFN-γ in controlling the bacterial load after infection. Furthermore, the infection was greatly exacerbated by anti–IL-18R mAb even in IFN-$\gamma^{-/-}$ mice. Finally, IL-18 operated independently of IFN-γ to reduce the bacterial load early in infection. Although these findings are consistent with the IFN-γ–dependent role of IL-12 in protection to a primary challenge with *Listeria* (16) they suggest an additional role for IL-18. We had originally published findings describing the mechanism by which IL-18 induced IFN-γ production by Th1 cells in concert with IL-12 (34). Moreover, many studies now suggest that the ability of IL-18 to enhance cell-mediated Th1-type responses is mainly via the upregulation of the IL-12 receptor (70, 71). Our present findings, showing that IL-18 has effects over and above the induction of IFN-γ, demonstrate its more global role in immune responses than had originally been suggested. That IFN-γ levels remained detectable after anti–IL-18R mAb treatment also indicated that the responsive cells were not being depleted by this mAb.

We show that IL-18 and TNF display a similar capacity to protect mice from *Listeria* and that the protection mediated by IL-18 can only partially overcome the ability of

anti-TNF to enhance the bacterial load, thereby demonstrating that IL-18 mediates protection to a large extent through TNF action in vivo. Since we were unable to demonstrate a requirement for IL-18 in the TNF produced by in vitro cultures designed to stimulate T and NK cells from *Listeria*-infected mice we investigated the macrophage as an alternative IL-18–dependent source of TNF. Indeed, we found that TNF production by F4/80$^+$ peritoneal macrophages was induced by IL-18 after a *Listeria* infection, in keeping with previous findings in nonpathogen-driven systems (62–64). Moreover, our ability to show that the TNF produced in response to HKLM was IL-18–dependent provided further evidence for the TNF-inducing properties of IL-18 in response to *Listeria.* Hence we conclude that the role of IL-18 in responses to *Listeria* encompasses the induction of TNF production by macrophages and IFN-γ production by antigen-specific T cells and NK cells.

Our findings showing that macrophages were triggered by IL-18 to secrete TNF in response to *Listeria* led us to speculate that other properties of the macrophage may also be IL-18 dependent. We confirm here that IL-18 is required for the macrophage's subsequent release of NO in response to a *Listeria* infection in vivo. Since NO production is a key mediator of the macrophage's listericidal effects (65–67), this may be a major mechanism to explain the profound influence of IL-18 on bacterial clearance.

We also show that upon neutralization of IL-18, *Listeria* elicited a massive neutrophil influx to the liver with formation of multiple microfoci. This pathology was comparable to that seen in the absence of TNF with fewer and smaller microfoci observed in the absence of either IL-12 or IFN-γ. Hence, the situation in the liver correlated with the relative capacity for bacterial clearance from the spleen in these situations. It has been shown that a "spillover" of *Listeria* from the granuloma to neutrophilic microabscesses occurs in SCID mice chronically infected with *Listeria* and depleted of IFN-γ or TNF (72). In our hands, a lack of IL-18 receptor signaling resulted in a similar outcome but in this case in immunocompetent mice, reflecting an inability to clear the organism and a disruption in the equilibrium between the macrophage-rich granulomas and the neutrophilic microabscesses.

Here we show for the first time that in addition to its protective role in primary immunity to *Listeria,* IL-18 is as important in memory effector responses to reinfection with *Listeria.* Since IL-18 was required for optimal levels of IFN-γ it may represent the missing element which has been postulated to act in combination with IL-12 for maximal IFN-γ recall responses to *Listeria* (28). Hence, one role for IL-18 may be to induce optimal Th1 memory effector cell responses to repeat infections with *Listeria.* However, in our hands IL-18 appeared to have a more extensive role in bacterial clearance than via its effects on IFN-γ. Indeed IL-18 was as potent as TNF in bacterial clearance. Effective recall responses to *Listeria* are known to require TNF (27, 29) but its mechanism of induction and subsequent effects remain to be clearly defined in a recall response to *Listeria.* The extent to which IL-18 operates via TNF and/or other additional mechanisms in a secondary infection also remains to be determined. As these findings were reminiscent of those observed during a primary immune response to *Listeria* and indeed the histology was also similar, it seems possible that similar mechanisms may operate to eradicate intracellular pathogens during primary and memory effector responses, with IL-18 playing a dominant role at both stages.

It is also possible that IL-18 influences other responses and/or cell types invoked by a *Listeria* infection. IL-18 can augment the cytotoxic activity of CD8$^+$ T cells (73) and this cell subset is an essential component of the host defense to *Listeria* (40). The rapid mortality of *Listeria*-infected mice given anti–IL-18R showed that IL-18 was important for controlling bacterial expansion at an early stage in the infection. Indeed we found that mice treated with anti–IL-18R did not survive beyond days 3–4 (Fig. 1) even after infecting with only 100 CFU *Listeria* (data not shown). Since a primary CTL response to *Listeria* is not detectable until after this time (74, 75) we were unable to determine how its induction was affected by anti–IL-18R mAb treatment. Memory effector CTLs on the other hand are invoked more quickly to *Listeria* (74, 75), and we show here that IL-18 is also required for an effective recall response to secondary challenge with *Listeria.* However, we could find no impairment of the antigen-specific CTL responses during a secondary challenge in *Listeria*-immune mice treated with anti–IL-18R before reinfection (data not shown). Therefore, we can conclude that IL-18 is not required for the cytotoxic effector function of memory CTLs induced by a secondary infection with *Listeria.* However, the bacterial load is very high (Fig. 7 A) when *Listeria*-immune mice are given anti–IL-18R mAbs before reinfection suggesting that the CTL activity may be insufficient for host resistance in the absence of effective innate immunity. Moreover, the possibility still exists that IL-18 is required for the development of memory CTLs. This will be difficult to assess since mice will not survive treatment with anti–IL-18R mAbs during a primary infection to allow the development of memory CTL responses to *Listeria.*

IL-18 has been shown to be important for organism clearance in a number of acute pathogenic infections. IL-18$^{-/-}$ mice were impaired in their capacity to eliminate *Leishmania major* (46) and *Shigella* (47). Moreover, anti–IL-18 treatment exacerbated the bacterial burden after infection with *Salmonella* (48) or *Yersinia* (44), while IL-18 protein treatment reduced titers of herpes simplex virus type 1 (45) and lowered pock formation after a *Vaccinia* virus infection (49). Only this latter study suggested an IFN-γ–independent protective role for IL-18 by showing that IL-18 treatment improved symptoms even in mice infected with *Vaccinia* virus and depleted of IFN-γ. The extent of IFN-γ–independent IL-18–mediated protection had not, to our knowledge, been investigated in bacterial infections. Moreover, only the role of IL-18 in the acute response to an initial infection had been focused on in the existing literature, in contrast to our study demonstrating a role for IL-18 in both primary and recall responses to *Listeria* infections.

In conclusion, our study shows that IL-18 plays a profound role in protective cell-mediated immune responses to an intracellular pathogen, which extend beyond its known effects on the induction of IFN-γ by NK and T cells, to the induction of TNF and NO production by macrophages. For these reasons we believe that our data have important therapeutic implications for the use of IL-18 to induce appropriate immune responses during vaccination or to eradicate chronic infections arising as a result of immunosuppression. Conversely, it is known that such responses can also be pathology-inducing in autoimmune and inflammatory disorders and indeed IL-18 has been implicated in the development of several autoimmune diseases (76–80). Therefore, our data would predict that antagonizing the global effects of IL-18 might have more impact on the treatment of such diseases than the current strategies of TNF, IL-12, or IFN-γ antagonism. Our novel finding that IL-18 is necessary for a recall cell-mediated immune response further supports the potential for using IL-18 antagonists in preexisting inflammatory pathologies.

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