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Mice Deficient in LRG-47 Display Increased Susceptibility to Mycobacterial Infection Associated with the Induction of Lymphopenia

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Although IFN-γ is essential for host control of mycobacterial infection, the mechanisms by which the cytokine restricts pathogen growth are only partially understood. LRG-47 is an IFN-inducible GTP-binding protein previously shown to be required for IFN-γ-dependent host resistance to acute *Listeria monocytogenes* and *Toxoplasma gondii* infections. To examine the role of LRG-47 in control of mycobacterial infection, LRG-47−/− and wild-type mice were infected with *Mycobacterium avium*, and host responses were analyzed. LRG-47 protein was strongly induced in livers of infected wild-type animals in an IFN-γ-dependent manner. LRG-47−/− mice were unable to control bacterial replication, but survived the acute phase, succumbing 11–16 wk postinfection. IFN-γ-primed, bone marrow-derived macrophages from LRG-47−/− and wild-type animals produced equivalent levels of TNF and NO upon *M. avium* infection in vitro and developed similar intracellular bacterial loads. In addition, priming for IFN-γ production was observed in T cells isolated from infected LRG-47−/− mice. Importantly, however, mycobacterial granulomas in LRG-47−/− mice showed a marked lymphocyte deficiency. Further examination of these animals revealed a profound systemic lymphopenia and anemia triggered by infection. As LRG47−/− T lymphocytes were found to both survive and confer resistance to *M. avium* in recipient recombinase-activating gene-2−/− mice, the defect in cellular response and bacterial control in LRG-47−/− mice may also depend on a factor(s) expressed in a nonlymphocyte compartment. These findings establish a role for LRG-47 in host control of mycobacteria and demonstrate that in the context of the IFN-γ response to persistent infection, LRG-47 can have downstream regulatory effects on lymphocyte survival.

an important role for LRG-47 in regulating lymphocyte dynamics during persistent infection.

**Materials and Methods**

**Mice**

LRG-47<sup>−/−</sup> and IGTP<sup>−/−</sup> mice on a C57BL/6J background were generated as previously described (22, 23). IFN-γ and IFN-γ receptor-deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and C57BL/10.recombinase-activating gene 2 (RAG2)<sup>−/−</sup> and wild-type (WT) B6129F<sub>1</sub> and C57BL/6 mice were obtained from Taconic Farms (Germantown, NY). The lines of LRG-47<sup>−/−</sup> and IGTP<sup>−/−</sup> mice, derived by intercrossing animals on a C57BL/6<sup>−/−</sup> background, were found to express the natural resistance-associated macrophage protein gene 1 (Nramp1) susceptibility allele, which leads to increased susceptibility to M. avium (24, 25). For this reason, we used both C57BL/6 (Nramp1-susceptible) and B6129F<sub>1</sub> (Nramp1 heterozygous) mice as controls in the majority of experiments involving M. avium infection. All mice were maintained at an Association for Assessment of Laboratory Animal Care-accredited animal facility at the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD). Mice of both sexes, between 8 and 12 wk of age, were used.

**Mycobacterial infections**

Mice were infected i.v. with 1 × 10<sup>8</sup> CFU of M. avium (Sm/i 2151 strain) or aerosolized M. tuberculosis (Erdman strain) in a Middlebrook airborne infection apparatus (Glas-Col, Terre Haute, IN). In the case of M. tuberculosis infection, each mouse received ~100 CFU measured in the lung at 24 h after exposure. Bacterial loads in infected organs were quantitated by culture on 7H11 agar as previously described (26).

**Cells and cell cultures**

Splenic T lymphocytes were isolated from naive or M. avium-infected WT control or LRG-47<sup>−/−</sup> animals using T cell enrichment columns (R&D Systems, Minneapolis, MN). Isolated T cells (1 × 10<sup>6</sup>/ml) and irradiated splenocytes (4 × 10<sup>6</sup>/ml) from naive WT animals were stimulated with medium, plate-bound anti-CD3 (10 μg/ml), or soluble M. avium Ags (MA-Vag; 20 μg/ml) for 72 h in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μM glutamine, 10 mM HEPEs, and 50 μM 2-ME (complete RPMI). IFN-γ levels in culture supernatants were determined by ELISA as previously described (26).

Peritoneal exudate macrophages were elicited by i.p. injection of 1 ml of sterile thioglycollate medium (3%) and subsequently were harvested by peritoneal lavage 3 days after injection. The cells (10<sup>6</sup>/cell/well in six-well plates) were cultured in complete RPMI 1640 at 37°C, and 4 h later non-adherent cells were removed, and 5 ml of fresh antibiotic-free medium was added. The adherent cells were then used for analysis by Western blotting.

Bone marrow macrophages (2 × 10<sup>6</sup> cells/well), generated in complete RPMI supplemented with 20% L929 cell culture supernatant, were stimulated with 100 U/ml murine IFN-γ in antibiotic-free medium for 16 h and then infected with live M. avium (multiplicity of infection, 10:1) for 4 h, the adherent cells were washed twice, and fresh complete RPMI was added. Culture supernatants were collected 72 h after infection, and TNF-α and NO were measured using commercial TNF-α ELISA kits (R&D Systems) and Greiss assays, respectively. After removal of supernatants, M. avium-infected macrophages were lysed using 1% saponin. The cell lysates were cultured on 7H11 agar to determine CFU.

**Measurement of LRG-47 protein expression**

The production of LRG-47 protein in the peritoneal exudate macrophages and livers of naive or 4-wk M. avium-infected animals was determined by Western blotting using a polyconal rabbit serum generated against a unique peptide in the sequence of the protein (23).

**Flow cytometry and leukocyte counts**

The numbers of lymphocytes in lymphoid organs were calculated by multiplying viable cell counts by the percentage of lymphocytes determined by flow cytometry according to their forward and side scatter parameters. CD4<sup>+</sup> and CD8<sup>+</sup> T cells were identified with specific mAb (clones RM4-5 and 53-6-7, respectively; BD PharMingen, San Diego, CA). RBC, platelet, and differential WBC counts were performed on tail-snip samples bled into EDTA-containing tubes using an CELL-DYN (Abbott, Chicago, IL) automated analyzer.

**Lymphocyte transfer experiments**

RAG2<sup>−/−</sup> animals were reconstituted by i.v. injection with column-enriched splenic T cells (5 × 10<sup>5</sup>/mouse) from naive or 2-wk-infected mice and challenged i.v. on the following day with 1 × 10<sup>8</sup> CFU of M. avium. Hepatic bacterial loads were determined either 6 or 4 wk postinfection, respectively.

**Statistics**

ANOVA was employed to analyze the significance of differences in means between multiple experimental groups. The multicomparison significance level for the one-way ANOVA was 0.05. If significance was detected by one-way analysis, pairwise differences were evaluated using Fisher’s protected least significant difference ANOVA post-hoc test. Statistical significance was defined as p < 0.05.

**Results**

**IFN-dependent induction of LRG-47 in M. avium-infected livers and macrophages**

To determine whether LRG-47 is induced as a consequence of M. avium infection, the expression of LRG-47 protein was analyzed in the livers of naive and 4-wk-infected mice by Western blotting. The protein was produced minimally in naive WT mice, but was strongly up-regulated after infection. In contrast, no induction of LRG-47 was observed in IFN-γ receptor-deficient animals, confirming the dependence of its expression on IFN-γ (Fig. 1A). To further analyze the requirements for LRG-47 induction after M. avium infection, peritoneal exudate macrophages from IFN-γ- and LRG-47-deficient mice were exposed to live M. avium in vitro in the presence or the absence of IFN-γ, and the expression of the LRG-47 protein was determined by Western blotting. IFN-γ knockout (KO) mice were deliberately employed in this experiment instead of WT animals to avoid possible activation of elicited macrophages by endogenous IFN-γ. As shown in Fig. 1B, M. avium infection on its own failed to induce LRG-47 in vitro, whereas IFN-γ addition resulted in marked up-regulation of expression of the protein.

**FIGURE 1.** IFN-γ-dependent induction of LRG-47 in M. avium-infected livers and macrophages. Expression of LRG-47 protein in the livers of naive or 4-wk-infected LRG-47<sup>−/−</sup>, WT, or IFN-γ receptor<sup>−/−</sup> animals was determined by Western blotting. A similar pattern was observed in each of three animals assayed per group (A). Production of LRG-47 by macrophages in vitro was determined. Peritoneal exudate macrophages from either LRG-47<sup>−/−</sup> or IFN-γ-deficient mice were infected with M. avium in the presence or the absence of IFN-γ, and LRG-47 protein expression was assayed 16 h later by Western blotting. Duplicates of each condition are shown (B).
**LRG-47⁻/⁻** mice show enhanced susceptibility to M. avium infection

To investigate the contribution of LRG-47 to host defense against mycobacterial infection, LRG-47 and WT control (both C57BL/6 and B6129) mice were infected i.v., and the survival of the animals was monitored. All LRG-47⁻/⁻ mice succumbed between 11–16 wk postinfection (p.i.), whereas the WT B6 or B6129 animals showed 100% survival until wk 25 (Fig. 2A). Infected LRG-47⁻/⁻ mice showed increased bacterial loads in the liver (wk 2 and 4) and lungs (wk 4; Fig. 2B). The impaired bacterial control in LRG-47⁻/⁻ mice was also evident when liver (Fig. 2C) and lung (data not shown) were stained for acid-fast bacilli.

It has been previously shown that although IGTP⁻/⁻ mice are acutely susceptible to infection with the protozoan parasite *T. gondii*, they are completely resistant to *L. monocytogenes* (22, 23). In agreement with this observation we found that IGTP⁻/⁻ mice infected with *M. avium* fail to show increased mortality and display tissue bacterial burdens at 2 and 4 wk p.i. indistinguishable from those in infected WT control animals (data not shown).

**Macrophages from LRG-47⁻/⁻ mice respond normally to M. avium infection in vitro**

To determine whether LRG-47⁻/⁻ macrophages are able to respond to *M. avium* stimulation by producing proinflammatory mediators, the production of TNF (Fig. 3A) and NO (Fig. 3B) by IFN-γ-primed, bone marrow-derived macrophages from naive WT and KO mice was compared 72 h after in vitro infection. WT and KO macrophages produced equivalent levels of these proinflammatory mediators. Moreover, bacterial counts in the same WT and KO cell lysates were indistinguishable (Fig. 3C), suggesting that the enhanced susceptibility of LRG-47⁻/⁻ mice is not a direct result of a defect in macrophage activation after stimulation with *M. avium*.

**LRG-47⁻/⁻ mice display normal T cell priming, but defective granuloma formation, in response to M. avium infection**

Control of mycobacterial infection by macrophages is known to depend on IFN-γ-producing T cells. We therefore analyzed IFN-γ production by T lymphocytes isolated from infected WT and KO mice at both 2 and 4 wk p.i. and found that LRG-47⁻/⁻ T cells are clearly primed, as assessed by their production of the cytokine in response to in vitro restimulation with MAVAg at levels not significantly different from those produced by WT T cells (Fig. 3D).

As granuloma formation plays a critical role in restricting mycobacterial dissemination (27), we examined whether this tissue response is altered in infected KO animals. Hepatic granulomas in 4-wk infected LRG-47⁻/⁻ mice were noticeably less compact than the equivalent lesions in WT animals (Fig. 4A). Most strikingly, the typical lymphocyte cuffs surrounding hepatic granulomas of WT mice were largely absent in KO animals.

**Mycobacterial infection induces lymphopenia in LRG-47⁻/⁻ mice**

To determine whether the reduced lymphocyte content in the granulomas of LRG-47⁻/⁻ mice results from impaired tissue migration, lymphocyte levels were also examined in spleens and peripheral blood of the same infected animals. Although indistinguishable in appearance from WT animals before infection, spleen sections from 4-wk infected LRG-47⁻/⁻ animals displayed a striking depletion in lymphoid follicles (Fig. 4B) along with an accompanying reduction in the number of lymphocytes per organ (Fig. 4C) as well as total lymphocyte counts in peripheral blood (Fig. 4D). As shown in Fig. 4D, both CD4⁺ and CD8⁺ T cell levels were affected. Although peripheral blood monocyte counts were unchanged, the decrease in absolute lymphocyte counts was compensated for by a significant increase in neutrophils in the infected KO mice (4-wk infected C57BL/6 vs LRG-47⁻/⁻ mice, 0.34 ± 0.1 vs 0.93 ± 0.2 × 10⁶/μl, respectively). Thymic atrophy and decreased thymocyte numbers (Fig. 4F), anemia and thrombocytopenia (data not shown) were also evident at this stage. A moderate increase in propidium iodide-positive CD4⁺ (25.6 ± 2.5% in KO vs 9.8 ± 1.4% in WT) and CD8⁺ (4.4 ± 1.0% in KO vs 7.6 ± 1.6% in WT) T lymphocytes was observed in the spleens of LRG-47 KO mice at 8 wk of infection, suggesting that at least part of the observed lymphopenia is a result of cell death.

**FIGURE 2.** Increased susceptibility of LRG-47⁻/⁻ mice to *M. avium* infection. Survival of LRG-47⁻/⁻, WT control B6129, and C57BL/6 mice (n = 9–12/group) following i.v. *M. avium* infection. Data are pooled results from two separate experiments that gave similar results (A). Bacterial loads at 2 and 4 wk p.i. in lungs and livers of WT (□, B6129; □, B6) and LRG-47⁻/⁻ (■) mice (n = 4; mean CFU ± SD) The experiment shown is representative of two performed (B). Acid-fast bacilli in hepatic granulomas of 4-wk infected WT (□) and KO mice were detected by the Ziehl-Neelsen staining procedure. Arrows in WT section indicate individual *M. avium* bacilli, whereas arrowheads in KO section point to two clusters of bacilli (C). Original magnification, ×630.

**FIGURE 3.** *M. avium*-induced proinflammatory and IFN-γ responses are not impaired in the absence of LRG-47. IFN-γ-primed, bone marrow-derived macrophages from WT (B6) and KO mice were infected in vitro with live *M. avium*. Secreted TNF (A), NO (B), and intracellular *M. avium* loads (CFU per well; C) were determined 72 h later. The data shown are the mean (±SD) of triplicate cultures and are representative of two separate experiments performed. IFN-γ production in supernatants of enriched splenic T cells from 2- and 4-wk infected WT (B6) and KO mice stimulated in vitro with MAVAg for 72 h (D). The means (±SD) of three mice per time point are shown, and the data are representative of two separate experiments performed. None of the differences between WT and KO cultures shown in each of the panels of this figure was statistically significant.
FIGURE 4. Mycobacterial infection induces lymphopenia in LRG-47−/− mice. Hepatic (A; original magnification, ×400) and splenic (B; original magnification, ×100) sections from 4-wk M. tuberculosis-infected mice stained with H&E to visualize tissue lymphocytes. Alterations in the numbers of total splenic lymphocytes (C), splenic CD4+ and CD8+ T cells (D), peripheral blood lymphocytes (E), and thymocytes (F) of LRG-47−/− (○ and □), WT B6 (□), and B6129 (◇ and □) mice at the times indicated (C) or at 4 wk (D–F) after M. avium infection. The data shown are the mean ± SD of four to nine mice per group. The experiments shown are representative of two performed.

LRG-47−/− mice have recently been shown to be highly susceptible to infection with M. tuberculosis (28), a finding that we independently confirmed using animals exposed aerogenically to the Erdman strain of this pathogen (Fig. 5A). M. tuberculosis infection was found to induce a lymphocyte reduction similar to that observed in M. avium-infected KO mice, as evidenced by decreased cellularity in draining mediastinal lymph nodes (Fig. 5B) and thymus (Fig. 5C) 4 wk after aerosol exposure.

LRG-47−/− lymphocytes display normal survival and confer protection against M. avium in LRG-47-sufficient RAG2−/− recipients

To investigate whether the lymphocyte depletion observed in M. avium-infected LRG-47−/− mice results from a defect in lymphocytes themselves or depends on a defect(s) expressed in the non-lymphoid compartment, we transferred naive T lymphocytes from WT or KO donors into M. avium-infected LRG-47-sufficient RAG2−/− recipients and assayed bacterial growth at 6 wk p.i. RAG2−/− mice reconstituted with naive WT or KO lymphocytes showed equivalent control of infection at this time point (Fig. 6A). Similar results were observed when 2-wk infected WT or KO mice were used as T lymphocyte donors (Fig. 6B). When the numbers of

FIGURE 5. Increased bacterial loads and reduced tissue lymphocyte levels in LRG-47−/− mice infected with M. tuberculosis. LRG-47−/− and control B6129 animals (n = 4 mice/group) were infected aerogenically with 100 CFU of M. tuberculosis (Erdman strain), and pulmonary bacterial loads (A) and lymphocyte numbers in mediastinal lymph nodes (B) were determined 4 wk later. The data shown are the mean ± SD for each group of animals. Representative H&E-stained sections of thymic lobes from individual KO and WT control animals are shown (C) to document the decreased cellularity of lymphoid tissue from infected LRG-47−/− animals. Original magnification, ×25; inset enlargement of area shown in box, ×200.

FIGURE 6. RAG2−/− mice reconstituted with LRG-47−/− lymphocytes exert normal control of M. avium infection. RAG2−/− mice (n = 4/group) were reconstituted with splenic T lymphocytes isolated from naive (A) or 2-wk M. avium-infected (B) B6129 (WT) or LRG-47−/− (KO) mice or were left untreated (None). The animals were challenged with M. avium on the following day, and hepatic CFU was determined at 6 wk (A) or 4 wk (B) p.i. (n = 4; mean CFU ± SD). *, Differences in CFU between unreconstituted and reconstituted RAG2−/− mice that were statistically significant (p < 0.05) as determined by ANOVA. The numbers (n = 4; mean ± SD) of splenic CD4+ T cells in the reconstituted RAG2−/− mice described in B were assayed by flow cytometry (C). The difference in CD4+ T cell counts between WT and KO reconstituted mice shown was not statistically significant.
CD4+ T cells were determined in spleens of reconstituted RAG2−/− mice in the latter experiment, no significant difference was observed between recipients of LRG-47−/− and WT donor T cells (Fig. 6C).

Discussion

Previous studies have indicated that the 47-kDa GTPases, IGTP and LRG-47, play a major role in regulating IFN-γ-dependent host control of T. gondii and L. monocytogenes during the acute phase of infection (22, 23). The data presented in this study now demonstrate that although they display an increased bacterial burden, M. avium-infected LRG-47−/− mice are able to survive into the chronic stage of infection. In the absence of LRG-47, mycobacterial infection induces a state of lymphopenia, suggesting that GTPase may have profound downstream effects on lymphocyte dynamics. Beginning 4 wk after bacterial exposure, LRG-47−/− mice display severe lymphopenia that manifests in part in the formation of granulomas lacking the lymphocyte cuffs characteristic of WT lesions. Because lymphocytes are known to be required for the control of chronic M. avium infection (7), it is feasible that this depletion of lymphocytes at the site of bacterial replication is a contributing factor to the loss of host control of bacterial growth occurring in LRG-47−/− mice.

The induction of a lymphopenic state by M. avium has been noted previously (29, 30) in studies employing infection with bacteria strains more virulent than the SmT2 2151 isolate used in this study. This lymphocyte loss was tentatively shown to be linked to the robust IFN-γ response induced by the virulent strains (2, 29). In contrast, the lymphopenia observed in SmT2 2151-infected LRG-47−/− mice does not appear to result from an augmented IFN-γ response, because when assayed before the in vivo lymphocyte reduction, T cells from the infected KO animals did not show significantly increased production of this cytokine. Moreover, recent studies (Y. Belkaid, unpublished observations) indicate that LRG-47−/− mice infected with Leishmania major also display profoundly reduced lymphocyte numbers in both cutaneous lesions and draining lymph nodes similar to those observed in tissues of M. avium-infected KO animals. Together these observations suggest that LRG-47 may be a common regulator of lymphocyte survival and/or production in the immune response to persistent infection. As IFN-γ itself is known to be a potent stimulus for lymphocyte apoptosis (2, 31, 32), one unifying hypothesis is that LRG-47 is induced by IFN-γ to protect lymphocytes and other cells from death mediated by the same cytokine. That members of the 47-kDa GTPase family play a role in controlling cell survival is supported by a recent study showing that overexpression of IGTP inhibits apoptosis and promotes the viability of virus-infected HeLa cells (33). However, it is important to note that mycobacterial infection of LRG-47−/− mice also resulted in reduced lymphocyte numbers in thymi (Figs. 4 and 5) as well as spleen and lymph nodes. The above observation raises the possibility that the lymphopenia occurring in infected LRG-47−/− animals may result at least in part from defects in lymphocyte production. Preliminary experiments in which we noted reduced cellularity in bone marrow sections from infected LRG-47−/− mice (data not shown) are also consistent with this second hypothesis.

Although our results indicate a major effect of LRG-47 deficiency on lymphocyte dynamics, it is not yet clear whether this defect is intrinsic or represents a downstream consequence of abnormal handling of mycobacteria by infected macrophages. Although LRG-47−/− macrophages showed no obvious defects in their response to M. avium in vitro, it is still possible that in vivo these cells are defective in their restriction of bacterial growth or trigger downstream effects on lymphocyte survival that, in turn, lead to enhanced susceptibility to infection. Consistent with the latter possibility is our observation that LRG-47-sufficient RAG2−/− mice reconstituted with LRG-47−/− T cells are able to control M. avium infection and fail to display the defects in lymphocyte survival seen in intact KO animals. However, because the transfer experiments were performed in T lymphocyte-deficient hosts, it remains possible that the homeostatic proliferation occurring in that situation may have over-ride any lymphoproliferative defects inherent in the T cells themselves. Studies are in progress to better define the cellular targets of LRG-47 deficiency in the context of persistent intracellular infection and to determine whether this GTPase plays any intrinsic role in regulating lymphocyte activation or survival.

IGTP, another member of the IFN-induced, 47-kDa GTP-binding protein family, has previously been demonstrated to play a crucial role in host control of acute T. gondii, but not L. monocytogenes, infection. Interestingly, when IGTP−/− mice were infected with M. avium, no defects in host resistance or lymphocyte response were observed. This finding taken together with our previous data argue that LRG-47 has specific effects on host control of bacterial infection that are not shared by IGTP. At present, the mechanism underlying this interesting distinction in the effect of the 47-kDa GTP-binding proteins on host resistance to intracellular pathogens is unclear. Although more phylogenetically similar to each other than to T. gondii, mycobacteria and Listeria reside in different intracellular compartments and therefore need not share the same defense pathways. Further comparative studies of the roles of LRG-47 and IGTP in host control of different bacterial and protozoan infections and of the intracellular localization of these proteins in infected cells should provide important clues concerning the basis of this specific association between host effector molecule function and different groups of pathogens.

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References


