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Immunity Related GTPase *Irgm1* promotes expansion of activated CD4⁺ T cell populations by preventing interferon- γ -induced cell death

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Summary

Mice deficient in interferon- γ (IFN- γ) inducible immunity-related GTPase, *Irgm1*, display defective host resistance to a variety of intracellular pathogens. This increased susceptibility to infection is associated with impaired IFN- γ -dependent macrophage microbicidal activity *in vitro*. Here, we show that *Irgm1* also regulated the survival of mature effector CD4⁺ T lymphocytes by protecting them from IFN- γ -induced autophagic cell death. Mice deficient in both IFN- γ and *Irgm1* were rescued from the lymphocyte depletion and increased mortality that occurs in single *Irgm1*^{-/-} animals following mycobacterial infection. These studies reveal a feedback mechanism in the T_H1 response that limits the detrimental effects of IFN- γ on effector T lymphocyte survival while promoting the anti-microbial functions of IFN- γ .

Introduction

Robust expansion of antigen-specific peripheral lymphocytes through increased proliferation and survival is essential to maintain effector cell populations at a size sufficient to combat infection. Multiple pathways have been shown to regulate the persistence of activated lymphocytes. In the case of CD4⁺ T cells, duration and strength of T cell receptor (TCR) stimulation as well as expression of co-stimulatory molecules all play a role in promoting lymphocyte survival^{1–3} whereas activation of programmed cell death (PCD) pathways

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eliminates activated T cells. Homeostatic regulation of T lymphocyte survival is thought to be mediated primarily through the induction of apoptotic (type I) cell death^{4,5}. Autophagy, a biological process known to promote cellular homeostasis in response to nutrient starvation, is frequently observed in dying cells and has been implicated as a mechanism of death in variety of differentiated cell types^{6,7}. Nevertheless, the role of this type II cell death pathway in PCD has been controversial^{8,9} and there has been little direct evidence for its participation in the death of activated T lymphocytes^{10,11}.

Although CD4⁺ T cell effector functions are ultimately linked to their ability to accumulate at the site of infection and to produce different classes of antimicrobial cytokines, such as IFN- γ (<http://www.signaling-gateway.org/molecule/query?afcsid=A001238>), interleukin 4 (IL-4) and IL-17, the impact of the cytokine milieu on CD4⁺ T cell survival has only recently been appreciated^{12–14}. IFN- γ produced by activated T and NK cells is essential for host resistance to many bacterial, parasitic and viral pathogens and in addition plays a role in promoting T_H1 differentiation. Importantly, IFN- γ signaling in cultured CD4⁺ T lymphocytes is also known to be anti-proliferative as well as pro-apoptotic and therefore detrimental for cell survival^{15, 16}. Consistent with these *in vitro* findings, IFN- γ -deficient mice show increased numbers of activated lymphocytes in both infectious and autoimmune disease settings^{17,18}. However, even in the presence of high concentrations of IFN- γ produced during T_H1 responses, effector CD4⁺ T lymphocytes clearly accumulate at sites of infection, suggesting that activated CD4⁺ T cells possess strategies for escaping suppression by IFN- γ . Although down-regulation of IFN- γ receptor expression has been suggested as one mechanism that protects T_H1 lymphocytes from IFN- γ -dependent inhibition^{19,20}, this process occurs only in fully differentiated T_H1 cells and thus does not explain how developing T cells survive and proliferate in the presence of autocrine and paracrine IFN- γ during early stages of the response.

IFN- γ induces the expression of a broad array of genes in both hematopoietic and nonhematopoietic cells. A set of these genes strongly induced by the cytokine encodes the p47 kDa GTPase family^{21,22}. One family member, Irgm1 (also known as LRG47) has been shown to be essential for IFN- γ -dependent resistance to a wide variety of different intracellular bacterial and protozoan pathogens²³. Although Irgm1 clearly controls the fate of pathogens in IFN- γ -activated macrophages by promoting phagolysosome maturation and autophagy^{24, 25}, this is not its only function in host resistance. *Mycobacterium avium*²⁶ and *Trypanosoma cruzi*²⁷ infected *Irgm1*^{-/-} mice develop a profound pancytopenia, a defect that we have recently shown to be associated with impaired expansion of *Irgm1*^{-/-} hematopoietic stem and progenitor cell populations²⁸. However, these hematopoietic abnormalities are unlikely to be the sole explanation for the rapid development of lymphopenia seen in infected *Irgm1*^{-/-} mice. Adult-thymectomized mice do not show a profound depletion in effector CD4⁺ T cells for a prolonged period following mycobacterial infection²⁹, indicating that the existing peripheral pool can transiently sustain lymphocyte numbers without further input of progenitors. The latter observation suggested to us that lymphopenia observed in infected *Irgm1*^{-/-} mice could also be due to decreased survival of mature effector cells.

Here, we have addressed the role of Irgm1 in the IFN- γ dependent regulation of cell survival in the periphery. Focusing on CD4⁺ T lymphocytes we show that IFN- γ induced Irgm1 was critical for the survival of activated mature lymphocytes since in the absence of Irgm1, IFN- γ triggered the death of these cells. Importantly, the lymphopenia and mortality previously observed in *M. avium*-infected *Irgm1*^{-/-} animals was completely abrogated in *Irgm1*^{-/-}*Ifng*^{-/-} double-deficient mice. These findings reveal an unexpected regulatory function for Irgm1 in lymphocyte survival and suggest that the GTPase is essential for the peripheral expansion of immune effector cell populations during T_H1 responses to intracellular pathogens. Since in our experiments death of *Irgm1*^{-/-} CD4⁺ T lymphocytes was shown to

involve an autophagic rather than apoptotic mechanism, our findings also reveal an unexpected role for IFN- γ induced autophagy in PCD of activated T lymphocytes.

Results

Activated CD4⁺ T cells fail to expand in the absence of *Irgm1*

To analyze a mature leukocyte population that undergoes extensive proliferation during infection and plays a role in host resistance, we focused on the CD4⁺ T lymphocyte compartment of the *Irgm1*-deficient animals and examined whether mature CD4⁺ T cells from these animals exhibit defects in proliferation or survival following TCR triggering. Naive wild-type or *Irgm1*^{-/-} CD4⁺ T cells were sorted by flow cytometry and stimulated with agonistic CD3 monoclonal antibody (mAb) in the presence of irradiated syngenic wild-type splenocytes (Fig. 1a) or alternatively with irradiated allogeneic wild-type splenocytes (mixed leukocyte reactions) (Supplementary Fig. 1 online) and thymidine incorporation measured 3 days later. We found that *Irgm1*^{-/-} CD4⁺ T cells incorporated less thymidine than wild-type cells in response to both stimuli. To determine whether *Irgm1*^{-/-} CD4⁺ T cells exhibit similar defects in response to specific antigen, we crossed *Irgm1*^{-/-} mice with ovalbumin (OVA) TCR transgenic OT-II mice to generate *Irgm1*^{-/-} OT-II animals. When stimulated with splenic dendritic cells (DCs) pulsed with either OVA protein or peptide OVA(323–339), *Irgm1*-deficient OT-II cells again displayed substantially reduced thymidine incorporation (Fig. 1b). Further experiments were then carried out to determine whether this impaired thymidine uptake was due to reduction in viable T cell numbers or a defect in DNA synthesis. Kinetic studies revealed that *Irgm1*^{-/-} OT-II cells displayed decreased thymidine incorporation to OVA peptide at all time-points analyzed after 24h (Fig. 1c) and that this defect correlated with a steady decline of CD4⁺ T cell numbers in the cultures (Fig. 1d). However, the *Irgm1*^{-/-} cells showed unimpaired DNA synthesis as measured by BrdU at 72h incorporation at the single cell level (Fig. 1e). These lymphocytes also displayed normal up-regulation of CD25 and production of IL-2 (Fig. 1e,f). Importantly, biochemical analysis showed that *Irgm1* deficiency did not affect multiple TCR-dependent signaling events associated with the activation (tyrosine and ERK phosphorylation) and survival (I κ B α degradation for NF- κ B activation and Akt phosphorylation) of CD4⁺ T cells (Supplementary Fig. 2 online). In addition, supernatants from peptide-stimulated wild-type OT-II cultures failed to rescue the impaired thymidine incorporation of *Irgm1*^{-/-} cells, suggesting that this behavior did not result from a deficiency in the production or depletion of soluble factor (s) required for T cell expansion (data not shown). Finally, the *Irgm1*-dependent defect was observed only upon TCR triggering since *Irgm1*^{-/-} lymphocytes did not show reduced viability relative to wild-type cells when cultured in medium only (Supplementary Fig. 3a online). Together, these observations reveal that although dispensable for activation and DNA synthesis, intrinsic expression of *Irgm1* is essential for the expansion of CD4⁺ T lymphocytes following TCR stimulation.

Expansion defects of *Irgm1*^{-/-} CD4⁺ cells is IFN- γ -dependent

Irgm1 expression in most cell types previously studied has been shown to be interferon-dependent. We found that *Irgm1* protein expression in anti-CD3 and anti-CD28 stimulated CD4⁺ T cells from naive wild-type mice was inhibited in the presence of IFN- γ mAb (Fig. 2a), indicating that induction of the protein in activated CD4⁺ T lymphocytes required IFN- γ . As predicted from this finding, *Irgm1* was strongly induced in *Irgm1*-sufficient T_H1 but not T_H2 cells and its expression in the former T cell subset was maintained for at least 8 days after activation (Supplementary Fig. 3b). Although *Irgm1* was preferentially expressed in T_H1 cells, *Irgm1*-deficient CD4⁺ T cells exhibited unimpaired IFN- γ production in response to OVA peptide under either non-polarized or T_H1 conditions (Fig. 2b) and showed no defects in T_H1 or T_H2 differentiation *in vitro* (Supplementary Fig. 3c). However, we consistently observed decreased numbers of *Irgm1*^{-/-} CD4⁺ T cells upon activation as compared to wild-type cells

following primary (Figs. 1,2c) and secondary (Fig. 2d) peptide stimulation under non-polarized or T_H1 conditions (both associated with IFN- γ production). In contrast, expansion of OT-II cell populations under T_H2 condition was minimally affected by *Irgm1* deficiency. The failure of the OT-II cells to proliferate upon secondary stimulation was not due to impaired cell cycle progression but instead was a consequence of increased death of dividing CD4⁺ T cells as shown by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and propidium iodide (PI) co-labeling experiments (Fig. 2e).

The above experiments suggest that the survival defect of *Irgm1*^{-/-} CD4⁺ lymphocytes was associated with the presence of IFN- γ . We next examined the effect of IFN- γ blockade on antigen-driven CD4⁺ cell survival. OVA peptide stimulated *Irgm1*^{-/-} OTII cultures exhibited a reduction in the percentage of viable dividing cells (38%) relative to *Irgm1*-sufficient OTII cultures (71%). The addition of neutralizing IFN- γ mAb to the *Irgm1*^{-/-} OTII cultures resulted in an increased accumulation of dividing cells (71%) with a concurrent reduction in the percentage of dead PI⁺ cells from 27% to 15% (Fig. 2f). To confirm this finding in a setting in which IFN- γ is completely absent, we crossed *Ifng*^{-/-} and *Irgm1*^{-/-} mice to generate animals deficient in both genes. Sorted naive wild-type, *Irgm1*^{-/-}, *Ifng*^{-/-}, *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells were stimulated with CD3 mAb in the presence of irradiated *Ifng*^{-/-} antigen-presenting cells (APCs) and thymidine incorporation assayed 72 h later. We found that in contrast to *Irgm1*^{-/-} cells, *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ lymphocytes exhibited unimpaired thymidine uptake comparable to the responses displayed by wild-type or *Ifng*^{-/-} CD4⁺ T cells (Fig. 2g). Together, these data established that IFN- γ inhibits expansion of activated CD4⁺ T cell populations in the absence of *Irgm1*.

Since in contrast to IFN- γ sufficient *Irgm1*^{-/-} CD4⁺ T lymphocytes, activated *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cell populations were able to undergo TCR-driven expansion *in vitro*, we were able to assess the role of *Irgm1* in the post-activation survival of CD4⁺ T cells *in vivo*. In these experiments, *in vitro* anti-CD3 activated *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells (CD45.2⁺) were adoptively transferred into wild-type congenic (CD45.1⁺) recipients previously infected with *Toxoplasma gondii* or *M. avium*, two pathogens known to potently induce IFN- γ responses. When the numbers of donor CD4⁺ T cells were compared in the peritoneum (Fig. 3a) or spleen (data not shown) 4 days post-transfer by flow cytometry, we found that while comparable cell numbers were recovered in naive recipients, *Irgm1*-deficient CD4⁺ T lymphocytes displayed decreased survival relative to *Irgm1*-sufficient cells in recipients infected with either pathogen, indicating that intrinsic expression of *Irgm1* in peripheral CD4⁺ T cells is required for their survival during pathogen-driven T_H1 responses.

Detrimental effects of IFN- γ in *M. avium*-infected *Irgm1*^{-/-} mice

To test the hypothesis that *Irgm1* protects lymphocytes and other cells from inhibition mediated by IFN- γ *in vivo*, wild-type, *Irgm1*^{-/-}, *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} C57BL/6 mice were i.v. infected with *M. avium* and survival, bacterial loads and hematological responses were monitored. While all *Irgm1*^{-/-} mice succumbed to the infection at approximately 40 days post-infection (p.i.), *Irgm1*^{-/-}*Ifng*^{-/-} animals displayed 100% survival throughout the course of these experiments (Fig. 3b). As described previously^{30,31} *Ifng*^{-/-} mice failed to show increased mortality during the same period. Although significantly higher than those in wild-type C57BL/6 mice, bacterial loads in *Irgm1*^{-/-}, *Irgm1*^{-/-}*Ifng*^{-/-} and *Ifng*^{-/-} animals were indistinguishable (Fig. 3c), indicating that the increased mortality of *Irgm1*^{-/-} mice cannot be explained solely by uncontrolled bacterial infection. Instead, in agreement with our previous findings²⁶ the decreased survival of infected *Irgm1*^{-/-} mice was associated with a marked lymphopenia as well as a less pronounced generalized pancytopenia (Supplementary Table 1 online). Nevertheless, CD4⁺ T cell activation was unimpaired since infected wild-type and *Irgm1*^{-/-} mice showed comparable increases in the percentage of activated CD44^{hi}CD62L^{lo}CD4⁺ T

cells in spleens prior to undergoing lymphopenia (Supplementary Fig. 4 online). Importantly, the subsequent reduction in total lymphocyte as well as CD4⁺ T cell counts was not observed in infected *Irgm1*^{-/-}*Ifng*^{-/-} or *Ifng*^{-/-} mice (Fig. 3d,e). Together, these data established that IFN- γ is essential for both the reduction in hematopoietic cells and early mortality of infected *Irgm1*^{-/-} mice.

The dependency on IFN- γ of the lymphopenia and mortality observed in these T_H1-skewed *M. avium* infected *Irgm1*^{-/-} mice suggested that *Irgm1* might play only a minimal role in *in vivo* host responses to T_H2-inducing helminths where IFN- γ production is suppressed³². To test this hypothesis, wild-type, *Irgm1*^{-/-}, *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} mice were infected with the parasitic trematode, *Schistosoma mansoni*, and host responses analyzed at 8 wks p.i. We found in contrast to our observations on *M. avium* infection, *S. mansoni*-exposed *Irgm1*^{-/-} animals showed no increase in mortality when compared to infected wild-type control animals (Supplementary Fig. 5a online). Moreover, *Irgm1*^{-/-} mice displayed normal circulating lymphocyte counts (Supplementary Fig. 5b) as well as other hematological parameters (data not shown). Importantly, schistosome-infected *Irgm1*^{-/-} mice showed unimpaired granuloma formation and tissue fibrosis, two well characterized T_H2-dependent pathological sequelae of infection³². Therefore, these data confirm *Irgm1* has no major influence on the development of host cellular responses in a T_H2 dominant setting.

IFN- γ directly triggers death of *Irgm1*^{-/-} CD4⁺ T cells

To characterize the mechanism of IFN- γ dependent suppression of *Irgm1*^{-/-} CD4⁺ lymphocytes, we examined the effects of exogenous IFN- γ on the division and death of anti-CD3 activated *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells. Irradiated *Ifng*^{-/-} APCs were used in these experiments to completely exclude endogenous production of the cytokine. We found that thymidine incorporation by *Irgm1*^{-/-}*Ifng*^{-/-} but not *Ifng*^{-/-} cells was profoundly inhibited in a dose-dependent manner by exogenous IFN- γ (Fig. 4a) and this inhibition was associated with reduced cell numbers in the *Irgm1*-deficient cultures (Fig. 4b), arguing that IFN- γ directly prevents the accumulation of CD4⁺ T cells. When T cell activation, division and viability of the above cultures were compared at 20 h, a time point prior to the first cell division, up-regulation of CD25 expression and PI staining were comparable in IFN- γ -exposed *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T lymphocytes, suggesting that IFN- γ does not inhibit the activation and survival of non-dividing *Irgm1*-deficient cells (Supplementary Fig. 6 online). After 48 h, *Irgm1*-sufficient and *Irgm1*-deficient CD4⁺ cells underwent similar numbers of divisions in the either presence or absence of IFN- γ . However, cell cycle progression in exogenous IFN- γ supplemented *Irgm1*^{-/-} cultures was accompanied by an increase in dead PI⁺CFSE⁺CD4⁺ T cell populations, which was clearly evident at 48 h (27%) and continued to accumulate, reaching ~50% of the total CD4⁺ T cells by 72 h (Fig. 4c). Thus, the enhanced death of proliferating cells appears to be a major factor limiting the expansion of *Irgm1*-deficient CD4⁺ T cell populations in the presence of IFN- γ . Consistent with this hypothesis, we observed that previously activated, IL-2 expanded *Ifng*^{-/-}*Irgm1*^{-/-} CD4⁺ lymphocytes are also highly susceptible to IFN- γ induced death (Fig. 4d). Since APCs were not present in the latter experimental setting, this observation further confirms a direct role of IFN- γ in the inhibition of *Irgm1*^{-/-} CD4⁺ T cell survival.

The increased IFN- γ -dependent death of *Irgm1*^{-/-} cells cannot be explained by augmented cytokine signaling as we found that the duration and magnitude of STAT1 phosphorylation in either fresh isolated (Supplementary Fig. 7a online) or IL-2 expanded (Supplementary Fig. 7b) *Irgm1*-deficient CD4⁺ T lymphocytes was indistinguishable from wild-type cells. To address whether *Irgm1*^{-/-} CD4⁺ T cells were more sensitive to TCR-induced cell death (TICD), we examined survival of IL-2 expanded CD4⁺ T cell populations upon restimulation with anti-CD3 and found that both *Irgm1*^{+/+} and *Irgm1*^{-/-} CD4⁺ cells showed comparable susceptibility

to TICD (Fig. 4e). In addition, *Irgm1*-deficient cells showed normal Fas and Fas ligand expression (data not shown) and were not more sensitive to Fas-dependent death signals than their wild-type counterparts in the presence or absence of IFN- γ (Fig. 4f). Therefore, neither TICD nor Fas plays a substantial role in the death of IFN- γ -treated *Irgm1*^{-/-} CD4⁺ T cells.

Irgm1 prevents IFN- γ -induced autophagic death of CD4⁺ cells

To investigate the mechanism of IFN- γ -induced cell death in *Irgm1*^{-/-} cells, we employed transmission electron microscopy to compare the morphology of IL-2-treated CD4⁺ T cells from *Ifng*^{-/-} and *Irgm1*^{-/-}*Ifng*^{-/-} animals following addition of IFN- γ to the APC-free cultures. Although a few *Irgm1*^{-/-} cells displayed evidence of end-stage apoptotic death as indicated by condensed chromatin and bleb-like structures, we observed many otherwise healthy appearing cells with significantly increased numbers of membrane-bound vacuoles closely resembling autophagosomes and autolysosomes in their morphology (Fig. 5a-d). Some *Irgm1*^{-/-}*Ifng*^{-/-} cells contained large number of vacuoles of diverse size and electron density (Fig. 5e) while others showed a near complete absence of cytoplasmic components (Fig. 5f). Further evidence for autophagosome formation was obtained in experiments analyzing the aggregation of microtubule-associated protein 1 light chain 3 (LC3) in IFN- γ -exposed *Irgm1*^{-/-}*Ifng*^{-/-} cells transiently transfected with LC3 fused to green fluorescent protein (GFP-LC3) (Supplementary Fig. 8 online). In direct contrast to these observations supporting an autophagic death pathway, we found that IFN- γ -induced death of *Irgm1*-deficient cells was not prevented by the addition of the pan-caspase inhibitor, zVAD, or other blockers of apoptotic cell death including zIETD, BocD and zDEVD (Supplementary Fig. 9a online). In addition, no reproducible differences were observed in the expression of Bcl2 family members (Bax, Bak, Bim, Bid, Bcl2 and Mcl1) with known pro- or anti-apoptotic functions between *Irgm1*^{+/+} and *Irgm1*^{-/-} cells in either the presence or absence of IFN- γ exposure *in vitro* (Supplementary Fig. 9b). In contrast, addition of the phosphatidylinositol-3-kinase (PI(3)K) inhibitors Wortmannin or Ly294002 led to a dose-dependent partial reduction in IFN- γ -induced death in *Irgm1*^{-/-} cell cultures (Fig. 5g), suggesting the involvement of PI3K-dependent autophagy in this process. Because the above pharmacologic agents have inherent toxic effects on the target cells and non-specifically inhibit other pathways, we further tested the involvement of autophagy by using siRNA to silence the expression of Beclin1 (ref. 33), a gene required for subcellular membrane condensation during the formation of autophagic vacuoles. We found that while knockdown of Beclin1 had no effect on the survival of *Ifng*^{-/-} cells, it significantly prevented IFN- γ -induced cell death in *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells (Fig. 5h). These findings argue that in the absence of *Irgm1*, IFN- γ -mediated cell death results primarily from autophagy and this involves a caspase-independent mechanism distinct from that previously described for IFN- γ -dependent death of *Irgm1*-sufficient CD4⁺ T lymphocytes¹⁶.

Discussion

IFN- γ induces a large array of genes involving diverse anti-microbial, anti-proliferative and immunomodulatory functions. However, the regulatory mechanisms that determine the beneficial versus detrimental outcomes of the IFN- γ response are poorly understood. Here, we show that *Irgm1*, previously characterized as a critical determinant of host resistance to many intracellular bacteria and protozoan agents and as an effector molecule required for macrophage microbicidal activity^{24,25}, plays a previously unrecognized role in IFN- γ -dependent susceptibility to infection by regulating the sensitivity of activated immune cells to suppression by the cytokine. Because *Irgm1* itself is strongly induced by IFN- γ , we propose that it provides a positive feedback mechanism in the IFN- γ signaling pathway that is designed to prevent the detrimental effects of the cytokine on cell survival while preserving its anti-microbial functions. Our data also reveal the existence of an autophagic death program in lymphocytes that is dependent on IFN- γ signaling and is clearly distinct from the autophagic cell death

mechanism previously described in macrophages which requires caspase inhibition and receptor interacting protein (RIP1) kinase induction^{7,34}.

We have previously reported that *Irgm1*^{-/-} mice develop profound lymphopenia associated with impaired self-renewal of hematopoietic stem cells following exposure to pathogens²⁸, suggesting impairment in lymphocyte replenishment. However, this defect alone cannot fully explain the rapid onset of lymphopenia occurring in infected *Irgm1*^{-/-} mice. We show here that depletion of mature effector cells could also contribute to the hematopoietic abnormalities observed in infected *Irgm1*^{-/-} mice. This increased cell loss in the periphery may also indirectly contribute to the observed lymphopenia by increasing the mobilization of progenitors from the bone marrow that would exacerbate hematopoietic failure. Therefore, we propose that *Irgm1* plays a major role in maintaining lymphocyte homeostasis during host IFN- γ responses by promoting the survival of effector cells. This activity of *Irgm1* is likely to be particularly important in the establishment and maintenance of T_H1 responses to persistent intracellular infections. Indeed, we failed to observe a similar function in a T_H2-driven infection (*S. mansoni*) *in vivo* and T_H2 polarized lymphocyte cultures *in vitro*. The above difference likely reflects the failure of *Irgm1* to be induced in the IFN- γ deficient setting of a T_H2 response. At a more general level, a regulatory mechanism comparable to that mediated by *Irgm1*, may not be necessary for T_H2 responses. While both IL-4 and IFN- γ are important polarizing factors for T_H differentiation, IL-4 in contrast to IFN- γ does not have the potential to suppress lymphocyte survival but rather serves as a T cell growth factor.

Both indirect and direct mechanisms have been proposed to explain the inhibition of lymphocyte proliferation or survival by IFN- γ ^{14,16,18,35,36}. An important indirect pathway involves the induction by the cytokine in APCs of nitric oxide and/or indoleamine 2,3-dioxygenase, two metabolic products that are thought to suppress both cycling and survival of activated T cells^{37,38}. Since *Irgm1* can shield T lymphocyte responses from IFN- γ mediated inhibition even in the absence of APCs, its major function is unlikely to involve protection from these reactive metabolites. Instead, the target of *Irgm1* regulation is more likely to be a process that protects T cell from direct inhibition by IFN- γ . Previously described mechanisms of this type include the down-regulation of IFN- γ receptor expression that occurs in differentiated T_H1 cells^{19,20} and the inhibition of IFN- γ induced STAT1 phosphorylation by TCR stimulation³⁹. Our observation that STAT1 phosphorylation occurred normally in IFN- γ stimulated *Irgm1*^{-/-} CD4⁺ T cells and was down-regulated following TCR engagement (data not shown) argues against both of the latter mechanisms. Similarly, although the induction requirements and regulatory activity of *Irgm1* resemble those of suppressor of cytokine signaling-1 (SOCS1), a known interferon-inducible regulator of IFN- γ signaling, *Irgm1* appears to exert distinct regulatory functions when compared to SOCS1. Thus, while both molecules promote host survival by preventing IFN- γ -dependent pathological responses⁴⁰ and are preferentially expressed in T_H1 CD4⁺ T cells⁴¹, *Irgm1*^{-/-} CD4⁺ T cells did not display the broad hyper-responsiveness (e.g., hyper-proliferation and dysregulated cytokine production) observed in their *Socs1*^{-/-} counterparts⁴². Moreover, the duration and magnitude of tyrosine phosphorylation of STAT1 was not enhanced in IFN- γ stimulated *Irgm1*^{-/-} CD4⁺ T cells, in direct contrast to the augmented STAT1 phosphorylation previously described for SOCS1 deficiency⁴³. Thus, the target of the IFN- γ -induced activity of *Irgm1* is likely to be either downstream of STAT1 or involve a JAK-STAT independent signaling pathway such as that mediated by PI(3)K^{44,45}.

IFN- γ has been shown to potentiate Fas-dependent TCR-induced cell death in an *in vitro* model in which the effect of IFN- γ was seen during TCR restimulation only in the absence of accessory cells¹⁵ and was shown to be dependent on caspase-8 activation¹⁶. In contrast, as shown here, repeated TCR ligation, a critical step for revealing the previously described inhibitory effects of IFN- γ on CD4⁺ T cell survival, is not required for cytokine-induced death of *Irgm1*^{-/-} cells.

Furthermore, *Irgm1*-deficient cells are not more sensitive to Fas-dependent TICD in either the presence or absence of IFN- γ . Instead, our study reveals that *Irgm1* exerts its major effects on CD4⁺ cell survival by regulating IFN- γ -dependent autophagic cell death. Although originally recognized as an essential cell survival response to starvation, autophagy has now been shown to play a more general role in maintaining cellular homeostasis^{46,47}. IFN- γ is a potent stimulus of autophagy in macrophages and plays an important role in the clearance of pathogens in these cells^{25,48}. In that setting, *Irgm1* has been shown to promote rather than inhibit autophagy as observed in the IFN- γ -stimulated T cells studied here. This distinction may reflect the fact that macrophages in contrast to CD4⁺ T cells are terminally differentiated non-dividing cells in which autophagy is utilized as a specialized anti-microbial effector mechanism in addition to its more generalized role in cellular homeostasis and survival. Interestingly, in macrophages autophagic cell death occurs only when apoptosis is inhibited^{7,34}. In contrast, we observe IFN- γ can induce autophagic death of *Irgm1*^{-/-} CD4⁺ T lymphocytes in the presence of intact apoptotic machinery. Thus, IFN- γ induced autophagy may play distinct roles in the innate and adaptive immune systems with *Irgm1* as a central regulator of this process exerting either positive or negative effects depending on its cellular context.

Our findings demonstrate that a single GTPase, *Irgm1*, can dictate the beneficial outcome of the IFN- γ response by providing a feedback mechanism essential for protecting activated CD4⁺ lymphocytes from IFN- γ -mediated death. It should be emphasized, however, that based on our previous work on the role of *Irgm1* in regulating the stress response of hematopoietic stem cells, the GTPase is likely to serve a similar function in other IFN- γ responsive cell lineages. Indeed, we have observed that the expansion of viral specific CD8⁺ T cells in lymphocytic choriomeningitis virus infected *Irgm1*^{-/-} mice is severely impaired (Feng and Barber, unpublished). Moreover, generalized effects of *Irgm1* on multiple differentiated leukocyte populations were observed in our previous studies and the rescue of their survival may in part explain the restoration in host resistance observed here in *M. avium* infected *Irgm1*^{-/-} mice in which IFN- γ is also absent. Taken together with these previous findings, our work argues that *Irgm1* plays a series of diverse interferon dependent functions in the immune response regulating cellular homeostasis as well as controlling the growth of intracellular pathogens. The common mechanism underlying these distinct activities is presently unclear although the data presented here suggest the autophagic process as a likely candidate worthy of further investigation.

Methods

Experimental animals

Wild-type C57BL/6 (CD45.2⁺) and C57BL/6.SJL (CD45.1⁺) as well as *Ifng*^{-/-} and OTII TCR transgenic mice bearing T cells reactive with peptides of OVA were provided by Taconic (Taconic Farms,) from the NIAID Animal Supply Contract. *Irgm1*^{-/-} mice back-crossed more than 12 times to C57BL/6 mice (kindly provided by Dr. G. Taylor, Duke University) were bred and maintained at the animal care facility at the NIAID. *Irgm1*^{-/-}OTII and *Irgm1*^{-/-}*Ifng*^{-/-} mice were generated by crossing *Irgm1*^{-/-} mice with TCR transgenic and *Ifng*^{-/-} animals, respectively. All mice were maintained in specific pathogen free conditions at an AALAC accredited animal facility at the NIAID, NIH (Bethesda, MD) under a study proposal approved by the NIAID Animal Care and Use Committee. Both male and female mice between 8 and 12 wk old were used.

In vivo infections—Mice were injected intravenously (i.v.) or intraperitoneally (i.p.) with 1 or 5×10⁶ colony forming units (CFU) of *M. avium* (strain SmT 2151) respectively and bacterial loads quantified as previously described²⁶. For *T. gondii* infection, mice were received 20 cysts of the parasite (ME49 strain) i.p.

Antibodies and flow cytometry

The following antibodies were purchased from BD Biosciences: fluorescein isothiocyanate-conjugated anti-B220 (RA3-6B2), anti-I-A/I-E (2G9), anti-CD49b (DX5), anti-CD44 (IM7) and anti-IFN- γ (XMG1.2); phycoerythrin-conjugated anti-V α 2 TCR (B20.1), anti-IL-2 (JES6-5H4) and anti-CD25 (PC61); Cy-Chrome-conjugated anti-CD4 (RM4-5); allophycocyanin-conjugated anti-CD62L (MEL-14), anti-CD45.2 (104) and anti-CD11c (HL3); Alexa Fluor 647-conjugated anti-IL-4 (11B11). Acquisitions were made with a FACS Calibur (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

Leukocyte counts

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⁺ T cells were identified with specific mAb by flow cytometry. RBC, platelet and differential WBC counts were performed on tail snip samples bled into EDTA-containing tubes using an automated analyzer.

Cell isolation and culture

FACS-sorted splenic naive CD4⁺ T cells (B220⁻DX5⁻I/A⁻CD62L⁺CD4⁺) were used in all *in vitro* experiments. FACS-sorted splenic DCs (B220⁻DX5⁻CD11c⁺) or irradiated total splenocytes from naive mice (with or without depleting Thy1.2⁺ cells by magnetic beads) were used as APCs. CD4⁺ cells (5×10^5 /ml) were activated with soluble CD3 mAb (145-2C11, 0.5 μ g/ml) or OVA peptide (0.1 μ M) in the presence of irradiated splenocytes (2×10^6 /ml) or sorted DCs (1.5×10^5 /ml) in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 10 mM HEPES pH 7.3 and 50 μ M 2-mercaptoethanol. Viable IL-2 expanded cells were enriched by Histopaque (Sigma) before being used in secondary restimulation or cell death assays. In some experiments, recombinant cytokines IL-4 (10 ng/ml), IL-12 (10 ng/ml) and IL-2 (10 – 50 U/ml) as well as mAb specific for IL-4 (11B11, 10 μ g/ml), IL-12p40 (C17.8, 10 μ g/ml) and IFN- γ (XMG 6, 10 μ g/ml) were included.

CD4⁺ T cell transfer

FACS-sorted naive CD4⁺ T cells from *Ifng*^{-/-} or *Irgm1*^{-/-}*Ifng*^{-/-} mice were activated with CD3 mAb as described above for 3 days then expanded in IL-2 for additional 4 days. 1×10^7 viable cells enriched by Histopaque (Sigma) were injected i.p. into C57BL/6.SJL (CD45.1⁺) mice that were infected i.p. with *T. gondii* or *M. avium* 3 days earlier. Peritoneal exudate cells (PEC) were harvested 4 days after cell transfer and donor cell were determined by flow cytometry following staining with the mAb to CD4, CD45.2, and CD3.

Measurement of cell expansion, division and cytokine production

Cell expansion was measured in triplicate by adding 1uCi/well ³H thymidine (ICN Pharmaceuticals) for the last 8 of 72 h culture. Alternatively, cells were pulsed with 10 μ M BrdU (BD Biosciences) during the last 4 of 72 h culture and intracellular expression detected by Flow cytometry using a BrdU detection kit (BD Biosciences). To follow cell division, cells were labeled with CFSE (Molecular Probes) before stimulation. CFSE dilution was determined by flow cytometry at 20, 48 or 72 h. Secreted IL-2 and IFN- γ in 72 h cultures were measured by ELISA.

Cell death assays and gene silencing

For cell death assays, anti-CD3 activated, IL-2-expanded CD4⁺ lymphocytes were seeded at 2.5×10^5 cells / ml in a 24-well plate and treated with IFN- γ (5 U/ml), anti-CD3 antibodies or

FLAG-tagged recombinant human CD95 ligand plus anti-FLAG antibody (Alexis) for 24 to 72 h. Cell viability was determined by flow cytometry with PI staining, in which all samples were adjusted to equal volume and acquired by a FACS Calibur (BD) at a constant time. The specific cell death is calculated based on PI-negative live cell counts: The % of cell loss = $(1 - \text{treated live cell counts} / \text{untreated live cell counts}) \times 100$. Pharmacological inhibitors Wortmannin and Ly294002 were purchased from Calbiochem (Calbiochem).

For silencing of Beclin1 gene, activated primary CD4⁺ T lymphocytes were treated with 50 U/ml IL-2 for 2 days and then subjected to siRNA transfection using Amaxa's mouse T cell kit and the nucleofector-II machine according to manufacturer's manual (Amaxa). The Stealth siRNA was obtained from Invitrogen (Invitrogen) with following sense and antisense sequences. Mouse *Beclin1*: 5'-AGCGGACAGUUUGGCACAAUCAUA and 5'-UAUUGAUUGUGCCAAACUGUCCGCU; Non-specific (NS) siRNA: 5'-AGCGACAGUUUCACGCUAAAGGAUA and 5'-UAUCCUUUAGCGUGAAACUGUCGCU. The transfected lymphocytes were cultured with supplemented mouse T cell nucleofector medium in the presence of 50 U/ml IL-2 for 2~4 days before further experiments.

Immunoblotting analysis of Irgm1 expression in primary CD4⁺ T cells

Lymphocytes were lysed with ice-cold TNT lysis buffer (30 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Triton X-100, and 1 × complete protease inhibitor cocktail, Roche) and Irgm1 protein expression was detected with a rabbit polyclonal anti-sera as previously described²⁶.

Electron Microscopy

FACS-sorted naive CD4⁺ T cells were activated and expanded in IL-2 as indicated above. Viable cells enriched by Histopaque (Sigma) were exposed to medium or IFN- γ (5 U/ml) for 30 h. Cells fixation and processing for the EM analysis were performed as described previously⁴⁹.

Statistics

Mann-Whitney test was used to determine the significance of differences between groups. *P* values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Irgm1^{-/-} CD4⁺ T cell populations fail to expand following TCR engagement. ³H thymidine incorporation of (a) wild-type (WT) or *Irgm1*^{-/-} CD4⁺ T cells activated with soluble anti-CD3 mAb and irradiated syngenic WT splenocytes, (b) OT-II or *Irgm1*^{-/-} OT-II CD4⁺ T cells stimulated with OVA protein or peptide in the presence of syngenic splenic DC, was measured at 72 h. Kinetic analysis of (c) ³H thymidine incorporation and (d) cell numbers in OVA (323-339) peptide stimulated OTII or *Irgm1*^{-/-} OT-II splenic cultures. (e) Flow cytometric analysis of T cell activation and proliferation. OVA peptide stimulated OT-II or *Irgm1*^{-/-} OT-II CD4⁺ T cells were pulsed with BrdU during the final 4 of 72 h culture and then co-stained with mAb to CD25 and BrdU. The numbers indicate the percentage of viable CD4⁺ cells. (f) IL-2 produced by OT-II (open bar) or *Irgm1*^{-/-} OT-II (closed bar) CD4⁺ T cells stimulated with OVA peptide in the presence of splenic DC measured at 72 h by ELISA. Data shown are the mean concentration ($\pm$ SD) of triplicate cultures. Results shown are the mean c. p. m. (a–c) or viable cell numbers (d) ($\pm$ SD) of triplicate cultures. Data are representative of more than five (a, b) or three (c–f) independent experiments with similar results.

Figure 2.

Irgm1 is essential for the expansion of CD4⁺ T cells in the presence of IFN-γ. **(a)** Immunoblot analysis of expression of *Irgm1* in WT CD4⁺ T cells. FACS-sorted naive CD4⁺ lymphocytes were activated with immobilized agonistic CD3 (10 μg/ml) and CD28 (2 μg/ml) mAb in the presence or absence of IFN-γ neutralizing mAb for 48 h and lysates prepared. **(b)** Concentrations of IFN-γ produced by OT-II (open bars) or *Irgm1*^{-/-} OT-II (closed bars) CD4⁺ T cells stimulated with OVA peptide in the presence of splenic DC under polarized or non-polarized (N) conditions in 72 h cultures were measured by ELISA. Data shown are the mean concentration (± SD) of triplicate cultures. ND: not detected. **(c)** Accumulation of polarized and non-polarized OT-II (open symbols) or *Irgm1*^{-/-} OT-II (closed symbols) CD4⁺ T cells following peptide stimulation quantified by viable cell count. **(d)** Viable cell numbers of OT-II (open bars) or *Irgm1*^{-/-} OT-II (closed bars) CD4⁺ T cells 72 h after restimulation with OVA peptide in the presence of T cell-depleted APCs. **(e)** Flow cytometric

analysis of viability and division of OT-II or *Irgm1*^{-/-} OT-II CD4⁺ T cells 72 h after secondary peptide stimulation. Peptide-stimulated OT-II or *Irgm1*^{-/-} OT-II CD4⁺ T cells were rested in IL-2 for 7 days, and then CFSE-labeled and restimulated with OVA peptide in the presence of T cell-depleted APCs. **(f)** Flow cytometric analysis of expansion of CFSE-labeled OTII or *Irgm1*^{-/-} OT-II CD4⁺ T cells activated with OVA peptide in the presence of absence of IFN- γ neutralizing mAb. **(g)** ³H thymidine incorporation of WT, *Irgm1*^{-/-}, *Ifng*^{-/-}, *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells stimulated with soluble agonistic CD3 mAb and irradiated syngenic *Ifng*^{-/-} splenocytes for 72 h. Results are the mean viable cell numbers **(c, d)** or c.p.m. **(g)** ($\pm$ SD) of triplicate cultures. FACS analysis was performed after gating on CD4⁺ T cells and the numbers indicate the percentage of CD4⁺ cells **(e, f)**. All data shown are representative of two independent experiments with similar results.

Figure 3.

Irgm1 promotes cell survival during pathogen-driven T_H1 responses and prevents IFN- γ -dependent mortality in mycobacteria-infected mice. **(a)** Survival of CD4⁺ T cells *in vivo*. CD45.2⁺*Ifng^{-/-}* (open symbols) or *Irgm1^{-/-}Ifng^{-/-}* (closed symbols) CD4⁺ lymphocytes pre-activated with agonistic CD3 mAb *in vitro* were transferred i.p. into congenic CD45.1⁺ C57BL/6.SJL recipients that were left untreated or inoculated i.p. with *T. gondii* or *M. avium* 3 days earlier. Percentage of donor CD4⁺ T cells in PEC collected at day 4 after transfer was determined by flow cytometry. **(b)** Survival of *M. avium* infected WT or KO mice ($n = 5$ per genotype) was monitored through the course of infection. **(c)** Splenic bacterial loads, **(d)** circulating and **(e)** splenic lymphocyte numbers were determined at wk4 after *M. avium* infection ($n = 5$). Data shown are representative of two independent experiments with each symbol representing an individual mouse.

Figure 4.

IFN- γ directly induces death of *Irgm1*^{-/-} CD4⁺ T cells. **(a)** ^3H thymidine incorporation. Naive CD4⁺ lymphocytes were stimulated with agonistic CD3 mAb and IFN- γ -deficient irradiated APCs for 72 h. IL-2 (10 U/ml) was added 24 h after initiation of the cultures. Data shown are the mean c.p.m. ($\pm$ SD) of triplicate cultures. **(b)** Accumulation of CD4⁺ T cells in the presence of IFN- γ (5 U/ml) at 72 h. Data shown are the mean viable cell numbers ($\pm$ SD) of triplicate cultures. **(c)** Flow cytometric analysis of cell viability and division. CD4⁺ T cells were CFSE-labeled and activated as in **(a)** with T-depleted *Ifng*^{-/-} APCs in the presence or absence of IFN- γ (5 U/ml). The numbers shown are the percentage of CFSE⁺CD4⁺ T cell populations in the cultures. **(d)** IFN- γ **(e)** agonistic CD3 mAb and **(f)** FasL triggered death of *Ifng*^{-/-} or

Irgm1^{-/-}*Ifng*^{-/-} T cells that were previously activated with anti-CD3 mAb and expanded in IL-2 medium. The cell loss was determined 48 h after each treatment with PI staining. In **a**, **b**, **d**, **e** and **f**, open bars: *Ifng*^{-/-}, closed bars: *Irgm1*^{-/-}*Ifng*^{-/-}. Data are representative of three independent experiments with similar results.

Figure 5.

Irgm1 prevents IFN- γ induced death of CD4⁺ T cells by regulating autophagy. EM analysis of (a) *Ifng*^{-/-} or (b - f) *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ lymphocytes that have been exposed to IFN- γ (5 U/ml) for 30 h. Original magnification: $\times 1000$. (c) A magnified view of the KO cell boxed in (b) with two autophagic vacuoles indicated with arrows heads; (d) the numbers of the vacuoles quantified blindly on 50 randomly selected EM sections with more than 400 cells. Each symbol represents one section; (e) a representative image of *Irgm1*^{-/-} CD4⁺ T cells with numerous vacuoles containing dense materials and (f) two *Irgm1*^{-/-} CD4⁺ cells with vacuolated cytoplasm. (g) IFN- γ -induced death of CD4⁺ T cells in the presence or absence of PI3K-inhibitors Wortmanin or Ly294002. Data are representative of three independent experiments. (h) IFN- γ induced death of *Ifng*^{-/-} or *Irgm1*^{-/-}*Ifng*^{-/-} CD4⁺ T cells transfected with control or Beclin1 specific siRNA. Reduction in expression of the Beclin1 protein was measured by

western blot. The data shown are means ($\pm$ SD) of triplicate cultures and are representative of three independent experiments. Cell death (**g, h**) was determined 48 h after IFN- γ exposure by flow cytometry with PI staining.