Coordinated, hierarchical loading of multiple IRG resistance GTPases on to the *Toxoplasma gondii* parasitophorous vacuole correlates inversely with parasite virulence.

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Abstract

The immunity-related GTPases (IRG proteins) constitute an interferon-induced intracellular resistance mechanism in mice against avirulent *T. gondii*. IRG proteins accumulate on parasitophorous vacuoles and participate in a process that leads to disruption of the vacuole and death of the parasite.

Accumulation of IRG proteins on the parasitophorous vacuole membrane (PVM) begins soon after parasite invasion and increases rapidly for about an hour. Loading is probably not initiated by an active signalling system and is probably diffusion-driven. Several factors, including the autophagic regulator, atg5, affect the timing and intensity of IRG protein accumulation. IRG

family proteins accumulate cooperatively on PVMs in an inclusion series reflecting a temporal hierarchy, with Irgb6 and Irgb10, apparently acting as pioneers. Loading of IRG proteins on to the vacuoles of type I virulent *T. gondii* strains is attenuated and the two pioneer IRG proteins are the most affected. The *T. gondii* polymorphic rhoptry kinases, ROP5, ROP16 and ROP18, are not responsible for the effect.

Thus IRG proteins can successfully protect the mouse against avirulent strains of *T. gondii*, but fail against virulent strains. Future work will determine the mechanistic and adaptive basis of both success and failure on both sides of this interesting host-pathogen relationship.

Introduction

Mice possess a powerful, interferon-inducible, cell-autonomous resistance mechanism against intracellular pathogens, the IRG proteins (immunity related or p47 GTPases). Mice deficient in single members of this large family are vulnerable to a number of intracellular pathogens (reviewed in [1,2,3,4]). Perhaps the most striking loss of resistance is seen after infection with avirulent strains of *Toxoplasma gondii*, where to date 5 IRG proteins have been implicated in resistance, either following gene disruption or in experimental systems *in vitro* [5,6,7,8,9]. There is no consensus about how IRG proteins exercise their resistance function. They have been implicated in rapid maturation of the *Mycobacterium tuberculosis* phagosome [10], in the initiation of an autophagic process in mycobacterial infection [11], and most relevantly to the present paper, in the direct disruption of the *T. gondii* parasitophorous vacuolar membrane (PVM) [6,8,9,12,13].

IRG proteins are strongly induced in many cell types by exposure to IFNγ [14]. In Western blot we have been able to detect expression in mouse embryonic fibroblasts within 3 hours of induction (Boehm, PhD thesis) and expression reaches a plateau between 24 and 48 hours after induction. The proteins distribute among distinct cellular compartments, Irgm1 to Golgi [15] and the endolysosomal compartment (Zhao, PhD thesis), Irgm2 to the Golgi [3,16], Irgm3 and Irga6 to the endoplasmic reticulum [15,17]. Native Irgb6 and Irgd are predominantly or wholly cytosolic ([15] and unpublished data) The 3 GMS sub-family members are largely or exclusively membrane bound [15]. After infection of IFNγ-induced cells with avirulent strains of *T. gondii*, 5 out of the 6 IRG proteins previously studied can be found in high density on the

PVM [6]. We show here that Irgb10 is also found on the *T. gondii* PVM. Irgm1 has not been reported on the PVM, but accumulates on phagocytic cups and phagosomes in cells phagocytosing *M. tuberculosis* or latex beads [6,7].

We previously showed in IFNγ-induced primary mouse astrocytes that the *T. gondii* vacuolar membrane becomes ruffled, vesiculated and ultimately disrupted, exposing the enclosed parasite to the cytoplasm, a process dependent on the presence of IRG proteins [6]. These findings have since been confirmed in mouse astrocytes [13] and peritoneal [8] and bone-marrow-derived [12] macrophages and mouse embryonic fibroblasts [9]. Thus there is no reason to believe that the disruption process is cell-type specific. Our recent experiments [9,18] strongly suggest that disruption of the IRG-loaded PV is the critical step in *T. gondii* restriction in IFNγ-induced cells. Disruption of the PV is invariably followed by the death of the parasite and shortly thereafter by the necrotic death of the infected cell [9]. The whole programme occurs within a few hours of infection and before tachyzoite replication is initiated. Virulent *T. gondii* strains, however, are not efficiently restricted by IFNγ induction, and this is correlated with reduced or absent vacuolar disruption and correspondingly reduced death of the parasites and of the host cells [9]. The reduced disruption of vacuoles containing virulent *T. gondii* is itself correlated with deficient loading of IRG proteins onto the vacuolar membrane [18,19].

In the present paper we examine various aspects of the process by which IRG proteins access the *T. gondii* PVM. Until now, this phenomenon has not been subjected to any quantitative analysis, despite its intrinsic interest, complexity and potential relevance to the resistance mechanism of the IRG proteins. We show that loading of IRG proteins onto the PVM begins almost immediately after infection, and rises rapidly for about an hour. There is a surprising heterogeneity in the intensity of vacuolar loading of IRG proteins, with some vacuoles remaining either completely or almost completely un-loaded. We show that the IRG proteins load onto the vacuole in a complex pattern, with Irgb6 and Irgb10 being the most efficient and also the pioneers in a temporal sense. We seek to explain how IRG proteins make their way from their normal cytoplasmic compartments to the vacuole, and conclude that diffusion from the cytosolic pools, rather than active transport, is the most likely route. Finally, we show in detail that the pattern of vacuolar loading is determined by the strain of *T. gondii* that inhabits the vacuole. The great majority of PVMs of virulent strains are far less intensely loaded with IRG proteins than those of avirulent strains. We present evidence that the virulence-associated polymorphic *T. gondii* kinases, Rop5, Rop16 and Rop18, are not responsible for this effect. Since the restriction

of virulent strains by IFNγ-treated cells is far less efficient than the restriction of avirulent strains this result confirms the general significance of the IRG proteins in resistance of mice to *T. gondii*, and places the relevant locus of action at the PVM itself.

Results

Rapid loading of the *T. gondii* ME49 PVM with IRG proteins in MEFs

We reported the accumulation of IRG proteins at the PVM of avirulent *T. gondii* strain ME49 at 2 h after infection in IFNγ-induced primary mouse astrocytes and other mouse cells [6,9,16]. In the present experiments we used primary mouse embryonic fibroblasts (MEFs) to document the time course of accumulation of IRG proteins at the vacuole. IFNγ-induced MEFs were examined for vacuolar IRG signals from approximately 2,5 minutes up to 2 hours after addition of *T. gondii* at a nominal multiplicity of infection (MOI) of 7 (Fig 1A). To increase the total signal intensity and thereby increase the probability of detecting weak IRG signals at the earliest time-points, cover-slips were immunostained simultaneously with two antisera directed respectively at Irga6 and Irgb6 and detected with second-stage reagents carrying the same fluorochrome. The fastest rise in the number of vacuoles accumulating IRG proteins under these conditions occurred from 15-45 minutes after infection, but a few vacuoles were already detectably loaded with IRG proteins at the earliest time-point of 2,5 minutes. The increase in number of detectably labeled vacuoles was also accompanied by an increase in the brightness of the fluorescent signals measured at the PVM (Fig 1B, filled circles, see materials and methods and Fig S1 for assay technique). This analysis also revealed large variation in the intensity of labelling of individual vacuoles. This variation was not apparently due to a wide asynchrony of infection times since there was no reduction in the intensity variation if free extracellular *T. gondii* were washed off after co-incubation for 15 min with the target cells (Fig 1B, open circles). We conclude from Fig 1 that there is no absolute lag phase before loading of the PVM with Irga6 or Irgb6 begins, and loading can be initiated within a minute or two of infection. Thus Fig 1A indicates an extremely rapid onset of PVM loading by IRG proteins followed by rapid intensification of the signals at individual vacuoles over time, slowing down from 30 minutes to 120 min post infection.

The rising phase of both the frequency and intensity data may be attributed to variation in the delay associated with loading individual vacuoles and to some asynchrony in the infection. We therefore used live-imaging and time-lapse photography to observe the loading of a transfected EGFP-tagged version of Irga6 (Irga6-ctag1-EGFP) on to individual PVMs. Fig 2A and Fig 2B show successive frames of two such videos (Fig 2A (Irga6 I in Fig. 2C) initiated immediately after addition of *T. gondii*, Fig 2B (Irga6 II in Fig. 2C) initiated 60 minutes after addition of *T. gondii*; the complete videos are at video S1 and video S2. In video S1 and Fig 2A, Irga6 on the PVM became detectable above the cytoplasmic Irga6 signal approximately 28 min after the addition of *T. gondii* and then rose continuously over about the next 30 min. The increasing intensity of signal at the PVM was accompanied by a reduction of cytoplasmic signal in the cell, suggesting that a significant proportion of the total available Irga6 was concentrated onto the PVM. In the second video (Fig 2B, video S2), a cell already containing one Irga6-loaded *T. gondii* vacuole (arrow) was invaded 60 minutes after infection by a second parasite as the video began (frame 1, arrowhead). This parasite began to accumulate Irga6 after about 9 minutes.

The signal intensities of Irga6-EGFP at the PVM in these two videos were quantified in consecutive frames (Fig 2C). Two further videos (not shown) showing the accumulation of Irgb6-FLAG-EGFP were also quantified. The four data sets illustrate two independent contributions to the rise in loaded vacuoles shown in Fig 1A and 1B. Firstly, there is variation in the time of entry of the *T. gondii* into the cell, which can be as early as the first minute or two in the Irga6 I series (Fig 2A) but also as late as 60 minutes in the Irga6 II series (Fig 2B). Secondly, there is heterogeneity in the length of the delay after infection before loading begins, which can be as short as 2,5 minutes in rare vacuoles seen in the fixed preparations (see Fig 1), and in one of the quantified Irgb6 videos (Irgb6 I) 6 minutes in Irga6 II (Fig 2B) and possibly up to 25 minutes in Irga6 I (Fig 2A). Once IRG accumulation is initiated it usually rises roughly linearly for 30 to 60 minutes.

These video series suggested the real existence of a delay of variable duration between infection and detectable IRG accumulation at the PVM (at least for Irga6 and Irgb6). This point was further analysed in a series of videos of cells doubly transfected with Irga6-ctag1-EGFP and Cherry as a cytosolic marker. Infection with *T. gondii* was detected in such cells by the appearance between consecutive frames of a dark shadow in the red fluorescent cytoplasm where Cherry is displaced by the incoming parasite [9]. In 7 videos of this type, the first detectable

Irga6-ctag1-EGFP at the PVM was seen at a mean of 10 minutes after infection with a range of 5-25 minutes (data not shown).

Heterogeneity of *T. gondii* PVM loading with IRG proteins

As noted, the combined intensities of vacuolar accumulation recorded for Irga6 and Irgb6 during the first 2 h after infection were remarkably heterogeneous (Fig 1B). A second experiment analysed pixel intensities of Irga6 and Irgb6 independently at the PVM in C57BL/6 MEFs induced for 12 to 48 h with IFNγ and infected for a further 2 h with *T. gondii* (Fig 3A, 3B). Absolute levels of Irgb6 and to a lesser extent Irga6 protein in the cells increased with time after IFNγ stimulation, as shown on the Western blot of cells sampled from this experiment (Fig 3C). In this analysis, 50 intracellular vacuoles defined by GRA7 staining at the PVM [20] were quantitated per time point for Irga6 and Irgb6. The great majority of vacuoles accumulated some IRG protein but the amount accumulated varied from very high values all the way down to the visible threshold and below. Fig 1B already suggested that very weakly or indeed unloaded vacuoles were not generally derived from "stragglers" which had only recently infected the cells. The mean expression of Irga6 and Irgb6 increased even up to 48 h after IFNγ induction (Fig 3C) and over this time span the mean intensity of IRG protein accumulation on the PVM of infecting *T. gondii* also increased (Fig 3A+B), suggesting that IRG protein accumulation on the vacuole is at least in part concentration-driven. However it is unlikely that heterogeneity in the intracellular concentration of IRG proteins is alone responsible for the striking heterogeneity of vacuolar loading since cell size-independent variation in intracellular Irga6 concentration in MEFs stimulated for 24 hours with IFNγ was over a range of less than 5-fold (data not shown), while the range of intensity of Irga6 on loaded vacuoles was up to 50 fold. We therefore conclude that most of the observed heterogeneity is intrinsic to the individual vacuole. This could be visualised directly for the cell shown in video of Fig 2B (video S2) which is already infected with one *T. gondii* (arrow) before a second one enters (arrowhead). The PVM of the first parasite already has a clear and intense Irga6-ctag1-EGFP accumulation. This does not change much during the course of the series while the accumulation on the PVM of the second *T. gondii* rapidly overhauls the first one and becomes very bright. Sometimes single vacuoles remained entirely free of IRG protein loading for hours while other vacuoles in the same cell could be heavily loaded (unpublished result). The nature of this vacuole-specific heterogeneity in IRG loading is

obviously of interest since it relates directly to the ability of individual organisms to escape attack by IRG resistance proteins.

Normal vacuolar loading of IRG proteins is independent of major signalling systems and of microtubules

We have shown that transfer of Irga6 to the *T. gondii* PVM shortly after infection in IFNγ-induced cells is accompanied by GTP-binding [16,21]. We therefore considered the possibility that the activation of Irga6 and transfer to the PVM results from the receipt of a cellular signal stimulated by infection. However disruption of major signalling systems failed to prevent the loading of IRG proteins onto the vacuoles of infecting *T. gondii*. We used wortmannin and Ly294002 to inhibit PI3 kinase, pertussis toxin to inhibit signal transmission from G-protein coupled receptors, MyD88-deficient MEFs to investigate a possible role of signalling through TLR signalling pathways and z-VAD to inhibit activation by caspases (see materials and methods for details). In no case was there any significant effect on the transfer of either Irga6 or Irgb6, analysed separately, to the PVM (Fig 4; for controls see Fig S2, A and B).

It was shown some years ago that Irga6 may interact with Hook3, a microtubule motor binding protein [22], raising the possibility that Irga6 and perhaps other IRG proteins are brought to the PVM by transport along microtubules. However dissociation of the microtubular network by nocodazole had no effect on the accumulation of IRG proteins at the PVM (Fig 4, and Fig S2 C).

At present, therefore, we consider that transfer of IRG proteins to the vacuole is probably mediated by diffusion from the known cytosolic pools of these proteins, while we argue elsewhere that activation of IRG proteins by GTP binding probably occurs at the PVM [16,21].

Normal loading of IRG proteins onto *T. gondii* parasitophorous vacuoles is partially dependent on the autophagic regulator, atg5

Mouse fibroblasts genetically deficient in the autophagic regulator, atg5, are deficient in their ability, following IFNγ induction, to restrict growth of type II avirulent ME49 strain of *T. gondii* [23]. Recently, [12] have shown that atg5-deficient macrophages stimulated with IFNγ and

LPS are deficient in accumulating Irga6 at the PVM of the type II avirulent PTG strain of *T. gondii*. We here examined in more detail IRG protein loading of avirulent ME49 vacuoles in atg5-deficient, transformed IFNγ-induced fibroblasts. The data shown in Fig 5A confirm [12] that fewer vacuoles in the atg5-deficient cells are loaded with Irga6. In addition, the loading intensity of individual vacuoles with Irga6 was somewhat reduced (Fig 5B), although some heavily loaded vacuoles were still present. However in the wild-type cells [12] found only about 10% of PV to be loaded with Irga6, while, as in Fig 5A, we routinely see 50%-70% loading of avirulent type II strain PVMs with Irga6. Furthermore we find that although accumulation of Irga6 onto the PV is reduced in atg5-deficient fibroblasts, it is not virtually eliminated as reported by [12] for atg5-deficient macrophages. Loading of Irgb6 was also significantly reduced in atg5-deficient cells, and that of Irgd almost absent, showing that the lesion is not specific for Irga6 (Fig 5A+B). A further difference from the results reported for macrophages by [12] is that the absolute levels of at least three IRG proteins, Irga6 and even more so Irgb6 and Irgd, were reduced in the transformed embryonic fibroblasts studied by ourselves (Fig 5C), while [12] observed no deficiency in Irga6 levels in primary bone-marrow macrophages. We found no quantitative deficiency of Irgm1 and Irgm3 in atg5-deficient fibroblasts, while Irgm2 was slightly reduced. [12] noted, Irga6 is at least partially distributed in small aggregates in IFNγ-induced atg5-deficient macrophages. We confirmed this result for fibroblasts and show (Fig 5D upper panels) that these aggregates are intensely stained with the monoclonal anti Irga6 antibody, 10D7, which, as we have recently shown, is specific for a conformation associated *in vivo* with the binding of GTP [21]. Irgb6 and Irgd were both also found in aggregates (Fig 5D middle and lower panels). Lastly, in our studies we were again unable to confirm in wild type (or atg5-deficient) fibroblasts a previous report [12] that *T. gondii* infecting wild-type macrophages co-localise with a LAMP1 compartment (Fig S3). In this respect our observations coincide with those of [6,9]. The quantitative deficiency in several components of IRG loading onto the PVM in IFNγ-induced atg5-/- cells leads to near complete loss of the *T. gondii* killing typical [9] of IRG-mediated resistance activity (data not shown).

IRG proteins accumulate at the PVM in a consistent hierarchy

Till now we have discussed loading of the PVM without considering the properties of individual IRG proteins in detail. Fig 3 has shown that Irga6 and Irgb6 load onto the majority of vacuoles.

Furthermore, the data shown in Figs 4 and 5A exemplify the general finding that Irgb6 invariably loads onto a higher proportion of vacuoles than Irga6, and indeed each IRG protein that we have analysed accumulates on a characteristic proportion of vacuoles. By double or occasionally triple immunofluorescence labelling we identified and discriminated two or three IRG proteins at a time on single vacuoles. We used this method to examine the proportions of loaded vacuoles, as well as the coupled or uncoupled distribution, of Irga6, Irgb6, Irgb10 Irgd, Irgm2 and Irgm3, for which we have antibody reagents that show saturation binding to their vacuolar targets (Fig S4). We have used a total of 4 immunoreagents to look for vacuolar loading of Irgm1 and conclude that this IRG protein, despite its strong association with resistance to *T. gondii*, does not localise at all to the vacuolar membrane (see also [6]). In order to identify intracellular organisms we observed the distribution of the dense-granule-derived protein, GRA7, which transfers to the PVM after infection, in addition to one or more of the IRG proteins. In case of co-staining for 3 IRG proteins, intracellular parasites were identified in phase contrast. Fig 6A shows that vacuolar loading by 5 IRG proteins fell into a consistent hierarchy in the order Irgb6>Irgb10>Irga6>Irgm2=Irgd. Irgm3 frequency is not shown. Irgm3 loads onto a small proportion of vacuoles but the loading intensity is also low and correspondingly difficult to resolve accurately from the surrounding ER to which Irgm3 is localised. The absolute values for each IRG protein fluctuated from assay to assay but the hierarchy was stable within each assay except for the relative positions of Irgd and Irgm2. Thus Irgb6 is at the top of the hierarchy whether determined by a commercial goat anti peptide serum (A20, Santa Cruz) or by a mouse monoclonal antibody (B34, [24]), Irgb10 is similar but slightly lower, while Irga6 is invariably lower than Irgb6 whether determined by a rabbit antiserum raised against bacterially purified protein (165, [15,25]) or by either of two mouse monoclonal antibodies (10D7, 10E7, [21]). Irgd-positive vacuoles were always found at a lower frequency than Irga6.

If IRG proteins load randomly onto individual vacuoles, then almost every vacuole should be loaded with one or the other and most with more than one. Since there are many other IFNγ-inducible IRG genes [26] in addition to those that we analysed, independent loading of each would guarantee heavy loading of all vacuoles with most IRG proteins. Surprisingly, however, this turned out not to be the case. When the loading of two or more members of the family was scored on each vacuole it was clear that they were strongly correlated. Fig 6B (see also Table S1) shows, for example, that all except 10 of 622 vacuoles loaded with Irga6 were also loaded with Irgb6, while all of 390 vacuoles loaded with Irgd were also loaded with Irga6. The two highest

members of the hierarchy, Irgb6 and Irgb10 were not quite so strongly correlated: nevertheless even here vacuoles loaded with both IRG proteins were significantly over-represented. The consequence of the strong correlation between different IRG proteins is that a significant number of vacuoles (normally 10-20%) did not load detectably with any IRG proteins at all while about 40% of the vacuoles (i.e. those loaded with Irgd or Irgm2) were loaded with all the IRG proteins we studied. It is important to recall that at least 7 further IFN-inducible IRG proteins have not yet been analysed, namely, Irga3, Irga4, Irga8 and 4 tandem IRGB proteins [26]. We have preliminary evidence that at least some of these also accumulate on the vacuolar membrane (data not shown) but their relation to the loading pattern recorded for the other IRG proteins has not yet been investigated.

The hierarchical loading behaviour suggested that IRG proteins might load onto the vacuoles in a temporal sequence. Independent analysis of Irag6 and Irgb6 accumulation on to ME49 *T. gondii* strain vacuoles in double-labelled samples taken shortly after infection showed that the earliest increase in label is due to accumulation of Irgb6, followed several minutes later by accumulation of Irga6 (Fig 6C). This result could be confirmed by live-cell observation of ME49 strain vacuoles in IFNγ-induced MEFs transfected with Irgd-ctag1-Cherry either together with Irgb6-FLAG-EGFP (Fig 6D) or with Irga6-ctag1-EGFP (Fig S5). Vacuoles loaded only with the "higher" member of the hierarchical series were observed in real time. At varying times after initiating the observations, the "lower" member was seen to accumulate (Fig 6D (Irgb6 followed by Irgd); see also supplementary Fig S5 (Irga6 followed by Irgd)). In no case were vacuoles loaded only with the "lower" member found. Thus the quantitative hierarchy of IRG protein loading is indeed also, at least in part, a temporal hierarchy.

If the loading succession is based on a formal "receptor-ligand" type of one-dimensional interaction, such that each member of the hierarchy binds exclusively with the member one stage higher and then provides an exclusive docking site for the member one stage lower, then we should expect that removing one of the higher members would block the loading of IRG proteins lower in the hierarchy. We therefore examined hierarchical loading in MEFs from Irga6-deficient C57BL/6 donors [6]. Removal of Irga6 did not appear to affect the loading of Irgd (or Irgm2, data not shown) to Irgb6-positive vacuoles: the hierarchical series formed as usual, but without Irga6 (Fig 6E). Furthermore the inclusion relationships described above were also preserved in Irga6-deficient cells (data not shown). It may be of interest, however, that there was a numerically highly significant tendency ($P<0.001$ by Mann-Whitney Test) towards reduced Irgb6 loading

intensity on Irga6-deficient vacuoles (Fig 6F), though not for Irgd or Irgm3. The formation of the loading hierarchy is evidently a complex dynamic process that we do not yet have a good description for.

Cooperative interactions in the loading of IRG proteins onto the *T. gondii* PVM

We have shown elsewhere that the 3 "GMS" proteins, Irgm1, Irgm2 and Irgm3, characterized by a unique substitution of methionine for lysine in the G1 motif of the nucleotide-binding site [14], are required to control the normal intracellular behaviour of the conventional "GKS" proteins, Irga6, Irgb6 and Irgd [16]. In the absence of the GMS proteins, Irga6 and Irgb6 form nucleotide-dependent cytoplasmic aggregates that are probably caused by premature GTP-binding and activation, resulting in the formation of oligomers [16,21]. We also observed that Irga6 was unable to relocate to the PVM of infecting *T. gondii* when expressed alone in mouse gs3T3 fibroblasts under the control of a promoter inducible by a synthetic hormone, but vacuolar location was qualitatively restored if the three GMS proteins were introduced as well by transfection. This result would argue against an essential role for Irgb6 or Irgb10 in Irga6 localisation at the vacuole. However we further observed that the frequency of loaded vacuoles was somewhat low under these conditions [16] and the accumulation of Irga6 at the PVM was abnormally weak (unpublished data). This suggested that other factors were indeed required for the full reconstitution of PVM loading and the hierarchical behaviour noted above further suggested that Irgb6 (and/or Irgb10) might contribute to this role. We therefore reconstituted Mifepristone-induced gs3T3-Irga6 fibroblasts with constructs expressing Irgb6, Irgd or both as well as the three GMS proteins and determined the Irga6 signal intensity on *T. gondii* PVM 2 hours after infection. Co-expression of either Irgb6 or Irgd or both caused a highly significant increase in the Irga6 signal intensity at the PVM although not significantly increasing the frequency of Irga6-loaded vacuoles (Fig 7, see also [16] and data not shown). Thus it indeed appears that the presence of other IRG proteins of the GKS group is a prerequisite for efficient Irga6 loading of the PVM. Irgd itself fails to load detectably on to the PVM if only the 3 GMS proteins are also present, but loads if Irgb6 is also present (data not shown). The equivalent result was observed for Irgm2 and Irgm3 [16]. These result, which are consistent with the hierarchy documented above, suggest that the loading of the PVM is a cooperative process, with Irgb6 and probably also Irgb10 arriving as pioneers, followed by Irga6, Irgd, Irgm2 and Irgm3 in

succession. In this context, it may be significant that both Irgb6 [16] and Irgb10 can load, albeit inefficiently, onto *T. gondii* vacuoles in the absence of other IRG proteins ([16]and Fig 7).

Reduced loading of IRG proteins onto the PVM of virulent strains

The data shown above was all based on infection of cells with the avirulent *T. gondii* strain, ME49. We have however observed that the restriction of replication of the virulent RH strain of *T. gondii* is inefficient in IFNγ induced MEFs and gs3T3 fibroblasts [9] and recent data has indicated a striking reduction of vacuolar loading of Irgb6 on RH PVs that correlated with reduced vacuolar disruption [9,18,19]. Fig 8A shows that the reduction of Irgb6 loading correlates directly with type I virulent strains. Two type I strains, BK and RH, gave grossly defective loading of Irgb6, while two avirulent type II strains, ME49 and NTE, and an avirulent Type III strain, CTG (data not shown), showed the characteristically high frequency of Irgb6-loaded vacuoles. Further analysis showed that loading of many IRG proteins was attenuated on RH strain vacuoles (Fig 8B), with Irgb6 and Irgb10 being much more affected than Irga6 or Irgd. More striking for these latter IRG proteins was the low intensity of the loading on RH vacuoles, documented for Irga6 in Fig. 8C. The few vacuoles that were visibly loaded with Irgb6 were loaded very heavily (Fig 8C+D) and these were additionally all intensely loaded for Irga6 (Fig 8E) and Irgd (data not shown). Irgb6-negative vacuoles were either not loaded or were only weakly loaded by Irga6 (Fig 8E) or Irgd (data not shown). The unusual patterns of IRG protein loading on vacuoles of Type I parasites are illustrated in Fig S6.

Thus virulent strains of *T. gondii* appear to interfere in a characteristic manner with the loading of the PVM by IRG proteins. We asked whether the infection of a fibroblast with a virulent *T. gondii* would result in interference with IRG loading of the PVM of a simultaneously infecting avirulent strain. We therefore co-infected IFNγ-induced MEFs with RH-YFP and unlabelled ME49 and examined the loading of Irgb6 onto individual vacuoles containing either ME49 or RH in doubly-infected cells. The failure of Irgb6 uptake by RH proved to be recessive in the sense that ME49-containing vacuoles could be intensely coated with Irgb6 while RH-containing vacuoles in the same cell had none (Fig 8F). Furthermore, the loading intensity of Irgb6 and Irga6 on ME49 PVs was not changed detectably in the presence of RH-YFP vacuoles (Fig 8G). It is therefore unlikely that the failure of Irgb6 loading is due to a freely diffusible

substance derived from the virulent strain that globally inhibits Irgb6 access to any PV in the same cell.

Recent studies have shown that several polymorphic *T. gondii* genes encoding rhoptry kinases of the ROP2 family contribute to the differential virulence phenotypes of *T. gondii* I, II and III type strains [27,28,29]. These proteins are secreted from the rhoptries into the host cell at the point of entry [30]. After secretion, ROP18 accumulates on the cytoplasmic surface of the PVM of the invading parasite and of previously invaded parasites, and this behaviour is paralleled by mature secreted form ROP18 expressed in infected cells by transfection [29]. ROP18 differs in amino acid sequence between all three virulence types [27], and is strikingly under-expressed in avirulent Type III parasites. It therefore seemed a plausible candidate to interfere with the IRG loading process. We expressed mature form ROP18 by transfection in IFNγ-induced mouse L929 fibroblasts infected with the avirulent ME49 strain. The expression of mature form ROP18 from the virulent (RH) strain had no effect on the number of vacuoles loaded with Irgb6 compared with similarly treated cells expressing either ROP18 of ME49 origin, or Cherry as a transfection control (Fig 9A). This result echoes that of [19] who showed that two independent clones of avirulent *T. gondii* Type III CTG strain expressing transgenic ROP18 from the virulent Type I RH strain, behaved like the avirulent host strain with respect to loading of Irgb6 in activated macrophages. Furthermore, in that study the virulent kinase had no effect on the ability of the activated macrophages to eliminate the transfected avirulent parasite.

Two further polymorphic ROP kinases have been implicated in virulence-related behaviour. ROP16 alleles have been shown to affect cellular signal transduction pathways differentially that are responsible for inflammation *in vivo* [31], though without large differential effects on virulence [27], while the ROP5 locus shows a strong association with virulence as measured by mice mortality ([27] and JC Boothroyd, unpublished). To find out whether either of these potential virulence determinants affected IRG protein loading we assayed the loading of Irgb6 onto the PVMs of virulent RH strain deficient in ROP16 (RH-GFP-Δ-ROP16) compared with the RH wild type control, and onto the PVMs of an avirulent strain, S22, transgenic for cosmid LC37 carrying the ROP5 kinase genes from the RH genome. Both of these genetic modifications have the anticipated effects on the in vivo behaviour of the modified strains. Thus the RH-Δ-ROP16 loses the characteristic pattern of phosphorylation of STAT3 and STAT6) while the S22 transgenic with ROP5 from RH gains at least 3 logs of virulence from the extreme avirulence of S22 (JC Boothroyd, unpublished). Neither genetically modified parasite was

affected in its behaviour vis-à-vis the cell autonomous IRG resistance mechanism (Fig 9B). RH-Δ-ROP16 behaved as a virulent parasite in that the PV showed the same deficit in Irgb6 loading as the wild type virulent RH strain. Likewise, S22 *T. gondii* transgenic with Type I virulent ROP5 alleles behaved like the avirulent S22 strain itself, the PVs loading at a high frequency with Irgb6 as well as Irga6. Both sets of strains behaved consistently in the uracil incorporation assay: RH-Δ-ROP16 was no better controlled by IFNγ treatment of the host MEFs than RH itself, while the ROP5 transgenic S22 strain was as well controlled by IFNγ as the wild-type S22 itself (Fig 9C). The untransformed data from these experiments are given in Fig S7.

Discussion

In this paper we have begun to document the process by which the parasitophorous vacuolar membrane (PVM) of *T. gondii* parasites infecting IFNγ-induced cells becomes coated with multiple members of the IRG family of interferon-inducible resistance GTPases. We showed that this process is important for resistance because IRG proteins are able to disrupt the PVM [6], and that vacuolar disruption leads to the rapid death of the enclosed parasite [9]. We also showed that the IRG-dependent resistance mechanism largely fails when the infecting *T. gondii* is a virulent type I parasite [9,18]. We now show that failure of the IRG system to control virulent *T. gondii* infections is due to a primary failure of the IRG proteins to load effectively on to the type I PVM. Thus a full understanding of the mechanistic basis for *T. gondii* virulence in mice requires an understanding of the process by which IRG proteins load on to the PVM.

The earliest infecting parasites begin to accumulate IRG proteins on the PVM within 2.5 minutes of addition to culture, but the proportion of IRG-loaded vacuoles rises for at least 45 minutes. Some of the variation in loading delay after infection certainly reflects asynchrony in the active infection of cells by the parasites since by live-cell imaging it is sometimes possible to observe a parasite enter a cell that has already been under observation for some time (Fig 2B and [9]). It is, however, also clear that there is a genuine delay of highly variable length before IRG proteins begin to accumulate. In the two videos shown in Fig 2 there are apparent delays of 20 minutes and 6 minutes respectively after cell penetration before Irga6-ctag1-EGFP protein becomes visible at the PVM. In several cases at least one intracellular vacuole failed to accumulate IRG proteins for hours (data not shown). However, once initiated, IRG protein accumulation is roughly linear for up to an hour (Fig 2C). These phenomena are consistent with

the idea that the initiation of accumulation has a low probability per molecule of IRG protein, while the accumulation of subsequent molecules has a high probability. Cytoplasmic Irga6 molecules are probably GDP-bound while those that accumulate on the PVM are GTP-bound [16,21]. *In vitro*, wild type GTP-bound Irga6 forms homo-oligomers [25] and extensive interactions between IRG proteins, documented elsewhere [16] and in the present study, would also allow for the formation of hetero-oligomers between distinct IRG proteins. One interpretation of the variable delay in initiating loading is that the rate-limiting step for colonisation of the PVM is the initiation of oligomerisation by GTP-binding at the PVM, while once oligomerisation begins, further molecules of GTP-bound IRG proteins can be added with high efficiency.

If loading of the vacuole is an autocatalytic process based on GTP-dependent oligomerisation all vacuoles might be expected to reach roughly the same level of IRG protein accumulation in the end. In fact there is great variation in loading intensity for both Irga6 and Irgb6 (Fig 1B, 3A+B), with a few vacuoles apparently failing completely to acquire any IRG proteins over many hours (Fig 2C, video S2, and data not shown). Is this a further reflection of the stochasticity of the initial lag phase or does it indicate a qualitatively different effect? One possibility is that the vacuoles "mature" after their formation and become progressively less accessible for IRG proteins, perhaps as a result of the expression of an inhibitory mechanism derived from the parasite. On this argument, vacuoles failing to load with IRG proteins early after parasite penetration would have a lower probability of loading later or would load to a reduced extent. This issue deserves further analysis since a "maturation" effect could indicate the existence of a parasite-determined process to counteract IRG proteins.

The hierarchy with which the different IRG proteins associate with the PVM is remarkable. Irgb6 and Irgb10 load with high efficiency, with a consistent but small advantage to Irgb6. Both load on to 70-80% of vacuoles and approximately 10% of vacuoles are loaded with only Irgb6 or only Irgb10 (Fig 6A and 6B and Table S1). Thus the great majority of vacuoles are loaded with either Irgb6 or Irgb10. Nevertheless, the loading of these two IRG proteins is apparently not independent, since there are always 10-20% of intracellular vacuoles that are negative for both IRGB proteins, more than expected by random allocation of the two proteins. Irga6 loads onto fewer vacuoles than either Irgb6 or Irgb10 but shows a strong preference for the majority subset of vacuoles that are doubly loaded with Irgb6 and Irgb10. Irgd, Irgm2 and Irgm3 all load less efficiently than Irga6, and with a near absolute preference onto vacuoles that are also

loaded with Irga6 (Fig 6B and data not shown). The data are consistent with the idea that the different IRG proteins assemble in a distinct order, with Irgb6 and Irgb10 as pioneers.

We showed recently that all three GMS proteins, Irgm1, Irgm2 and Irgm3, are required for loading of Irga6 onto the PVM of ME49 strain *T. gondii* ([16]). However this loading is inefficient unless other IRG proteins, Irgb6 and/or Irgd, are also present. The hierarchical loading data in the present study confirms this, since almost all, if not all, Irga6-loaded vacuoles are also Irgb6 and b10 loaded (Fig6B and Table S1), while the reverse is not the case. Finally, Irga6 loading of Irgb6-negative vacuoles containing the virulent RH strain is far less efficient than the loading of the rare Irgb6-positive vacuoles (Fig 8). The simplest interpretation of these results is that Irga6 accumulation is directly facilitated by the presence of Irgb6 and Irgd, and, we anticipate, of Irgb10 as well. It seems likely that this is due to the formation of mixed GTP-dependent oligomers because we have shown elsewhere that Irga6 and Irgb6 interact strongly in a nucleotide-dependent manner in yeast 2-hybrid assays [16]. Furthermore Irgb6 is largely co-localised in cytoplasmic aggregates with Irga6 in cells expressing a dominant-positive form of Irga6 that can bind but not hydrolyse GTP [6,16]. Consistent with this idea, the loading patterns observed for Irgb6, Irgd and Irgm2 on vacuoles in Irga6-deficient cells suggest that the hierarchy can be at least in part explained by mutually stabilising interactions between members of the GKS group of IRG proteins carrying the classical GTP binding site and probably all GTP-bound. Fig 6D shows a highly significant loss of signal for Irgb6 ($p<0,0001$) in the absence of Irga6. Our present thinking is that Irgb6 and probably also Irgb10 are "pioneers" in being able to load, albeit inefficiently, by themselves. Their loading is then stabilised by the arrival of the other GKS proteins. Whether such thinking is appropriate for the association of the GMS proteins with the PVM is not clear. The GMS proteins have low to absent free cytosolic pools [15] so access to the vacuole by free diffusion is improbable. Irgm3 is associated with the ER membrane, and it was proposed in a recent study that the association of this protein with the PVM is due to the investment of the PV by ER cisternae, rather than to a direct association of free Irgm3 molecules with the PVM [13], a position we also considered earlier [6] but were at that time inclined to reject. Irgm2 is specifically associated with Golgi membranes [3], but a secondary association with the PVM via Golgi-ER exchange is not excluded. Lastly, Irgm1, which is also associated prominently with the Golgi [7,15], has no detectable cytosolic pool [15] and is never detected on the PVM. The three GMS proteins stand in a special relation to the loading of the PVM that is distinct from the situation governing the GKS proteins. The GMS proteins function as essential

regulators of the GKS subfamily, maintaining these latter in the GDP-bound state in the cytoplasmic pool before infection [16]. It is certainly possible that GMS proteins have an independent effector function at the *T. gondii* PVM, and are directly required for some aspect of the vesiculation process. However another explanation for their presence at the vacuole is that this is a site of highly concentrated GKS proteins, initially in the GTP-bound state, but presumably exerting their function at the vacuole via GTP hydrolysis. Since at least Irgm3 has been shown to bind Irga6 in the GDP-bound state [16], it may be that Irgm2 and Irgm3 proteins at the vacuole reflect the binding of the small cytosolic pools of these proteins to PVM-localised GKS molecules that have just hydrolysed their GTP but have not yet dissociated from the vacuole.

Our experiments indicate that the transfer of IRG proteins to the PVM is not triggered via one of the principal cell-signalling systems activated by infection (Fig 4 and Fig S2). No effect was seen following inhibition of classical PI3 kinases, of G protein-couple receptors, of caspases, or by removal of the MyD88 adaptor of TLR signalling. The last is perhaps surprising in view of substantial evidence that the TLR system is stimulated by several independent *Toxoplasma* elicitors (reviewed in [32]). We have, however, not yet explored the possibility of a trigger emanating from a TLR via a MyD88-independent pathway or from a cytoplasmic detector such as one of the NALP proteins, so the conclusion that transfer of IRG proteins is untriggered is provisional. It also seems likely that the microtubule cytoskeleton is not directly involved in transporting IRG proteins to the PVM, despite published evidence that Irga6 interacts directly with the microtubule motor binding protein, Hook3 [22]. Depolymerisation of the microtubule cytoskeleton by nocodazole failed to block vacuolar loading by Irga6 and Irgb6 (Fig 4 and Fig S2). It may be that the interaction of Irga6 with Hook3 is more relevant to the destructive vesiculation events that follow the loading of the PVM with IRG proteins [6,8,13] rather than to the loading itself. The conclusion from these two kinds of experiments is that the infection process probably does not trigger the activation of IRG proteins. We would argue rather that it is the presence of the *Toxoplasma* vacuole in the cytosol that stimulates the relocalisation of IRG proteins. The key issue is, why do IRG proteins concentrate on this membrane, and in view of the hierarchy, one can perhaps simplify the question and ask, why do Irgb6 and Irgb10 accumulate on the PVM? It seems likely that Irgb6 initially reaches the PVM by simple diffusion. More than 70% of total cellular Irgb6 in the IFNγ-induced cell is freely cytosolic ([15] and unpublished results) and nothing is yet known about how Irgb6 associates with PV membranes. The protein

has no myristoylation motif, and we have shown that the myristoylation of Irga6 is essential for its own vacuolar targeting (unpublished results). Perhaps an early interaction with Irgb10, which has a perfect myristoylation motif [26], assists Irgb6 targeting. It may also be that Irgb6 and/or Irgb10 interacts with a specific receptor on the PVM, presumably derived from *Toxoplasma* itself during the entry phase, and that this interaction triggers uptake of GTP and activation. Certainly Irgb6 normally requires the presence of all 3 of the GMS proteins to avoid premature activation on cellular membranes [16]. The initial absence of GMS proteins on the PVM immediately after infection may therefore provide a window for spontaneous activation.

It was reported recently that IFNγ-induced mouse fibroblasts lacking the autophagic regulatory protein, atg5, are deficient in their ability to control avirulent *T. gondii* replication [23]. A possible explanation for this deficiency emerged with the demonstration that Irga6 fails to relocate to the PV in atg5 deficient macrophages [12], raising the possibility that atg5 has a specific function in loading Irga6 onto the PV. We show here that the deficiency in vacuolar loading in atg5-deficient cells extends to fibroblasts and includes Irgb6 and Irgd as well as Irga6. Confirming [12], we find that Irga6 forms aggregates in the atg5-deficient IFNγ-induced cells, and add that Irgb6 and Irgd are also aggregated. In view of these and other observations, we prefer not to attribute the loading defect of Irga6 to failure of a direct interaction between Atg5 and Irga6, as proposed by [12]. Rather, we propose that atg5 interferes with the regulation of GTP binding by the GKS subfamily of IRG proteins, normally controlled by dynamic interactions with the GMS subfamily. When this control is lost, Irga6 and Irgb6 form aggregates in the cytosol that fail to localise to the PVM [16]. Such aggregates bind the monoclonal Irga6-specific antibody, 10D7, which *in vivo* binds only to the GTP-dependent conformational state of Irga6 [21]. It is therefore significant that the Irga6 aggregates that form in atg5-deficient cells also bind 10D7. Furthermore, absolute levels of Irga6 and Irgb6 have been found to be reduced in IFNγ-induced fibroblasts from mice with disrupted Irgm1 or Irgm3 genes, and in these cells the two proteins also form aggregates [33], and we find Irga6 and Irgb6, as well as Irgd, but not the three GMS proteins, reduced in absolute levels in the atg5-deficient fibroblasts. As things stand, we consider it unlikely that atg5 is directly involved in vacuolar loading. It seems more likely that atg5 expression is necessary for the implementation of the function of the three GMS proteins in regulating nucleotide exchange in the GKS subfamily, which, we have argued elsewhere, probably occurs on the membranes of vesicular compartments. Which if any of the increasing range of activities attributed to atg5 [34,35] could be responsible for this activity is unclear.

The failure of Irgb6 and Irgb10 to initiate IRG loading on the great majority of vacuoles derived from virulent *T. gondii* strains speaks for some kind of specific inhibition by the parasite. It is curious that the two IRG proteins most affected by the virulence effect are Irgb6 and Irgb10, the two proteins that, of those tested, load earliest and most efficiently in the vacuolar loading hierarchy. If high virulence is an adaptive trait for *T. gondii*, which is far from obvious, the targeting of Irgb6 and Irgb10 would probably be an efficient way to implement a parasite defence strategy against IRG proteins, since in their absence vacuolar loading by other IRG proteins becomes inefficient. The inhibition of Irgb6 loading by the virulent strain is striking but not complete: approximately 10% of vacuoles load heavily with Irgb6, and these are also heavily loaded with Irga6. We proposed above that the failure of a proportion of vacuoles to load with IRG proteins even during infection with avirulent *T. gondii* could be due to some form of maturation towards resistance to IRG loading induced by the parasite. The same form of argument would also account for the exaggerated situation with virulent strains, perhaps because the hypothetical maturation process is quicker. Our findings in fibroblasts are similar to those recently reported by [19] in activated mouse peritoneal macrophages. These authors also report reduced IRG protein loading onto vacuoles of type I virulent *T. gondii*, but did not observe the minority of vacuoles with heavy IRG loading, perhaps reflecting the two rather different cellular systems.

The fact that inhibition is not dominant in mixed infections with virulent and avirulent strains suggests that the inhibitory effect must be mediated directly at the vacuolar membrane. Recently three polymorphic kinases, ROP5, ROP16 and ROP18, secreted from the rhoptries early in the penetration process, have been implicated in virulence differences between *T. gondii* strains [27,28,29]. ROP16 is highly polymorphic but its alleles are not associated with a strong virulence effect measured by early mouse mortality. The kinase profoundly influences pathways associated with inflammatory cytokine activity, but does not retarget the PV, is diffused in the cell and also carries a nuclear targeting signal [31]. It is therefore not an ideal candidate for the vacuole-specific loading deficit observed. Furthermore the ROP16 alleles of Type I and Type III strains are identical at the protein level [31], yet only Type I strains show the IRG loading deficit [18,19]. It is however relevant that Type II strain *T. gondii* transgenic for Type I or Type III ROP16 alleles showed a dominant reduction in virulence in the mouse mortality assay [27]. In the event, ROP16-deficient RH strain parasites showed the same IRG loading phenotype as RH itself (Fig 9B, C), suggesting that the IRG-related virulence property is not Type I ROP16

dependent. The case of ROP18 is different. The ROP18 protein products of all three clonal lineages are different, the natural targets of ROP18 in the cell are unknown and ROP18 is known to accumulate on the PVM of the newly invading parasite [29]. It is therefore in the right place to implement a vacuole-specific inhibitory action on IRG proteins. Our attempt to test this idea, by transfecting mature ROP18 from virulent type I RH-YFP in fibroblasts infected with the avirulent, type II Me49 strain, produced no detectable drop in the loading of IRG proteins onto the vacuole. [19], in a better approach, used transgenic avirulent Type III strain *T. gondii* expressing native ROP18 from a virulent type I strain with the same goal, and also recorded no reduction in the loading of Irga6 or Irgb6 onto the avirulent PVM. We therefore consider it unlikely that ROP18 kinase is responsible for the difference in virulence associated with differential loading of IRG proteins onto the PVM of virulent and avirulent strains. Lastly, ROP5 was found in a chromosomal domain associated with a strong virulence effect [27] and has also been shown recently to be associated with virulence: an avirulent recombinant strain S22-LC37, transgenic with a cosmid carrying the ROP5 locus from the virulent Type I RH strain, has greatly enhanced virulence in the mouse assay (JC Boothroyd, unpublished data). However, by the IRG-related assays described here S22-LC37 behaves no differently from the avirulent S22 strain.

We must therefore consider what aspect of virulence the IRG-related cellular assays measure. The association of IRG-related phenomena with highly virulent Type I strains is suggestive. However our data with those of [19] show that three genetically defined *T. gondii* proteins demonstrably associated with type I virulence properties fail to influence the IRG-related assays. In the case of ROP16 one may argue that the modification of inflammatory cytokine signalling associated with this ROP kinase is not closely associated with mouse mortality, and that the distribution of ROP16 alleles does not correspond to the distribution of virulence measured by mouse mortality. For ROP5, one may argue that a gain of 3 logs in virulence compared with the deeply avirulent S22 still does not reproduce the extreme virulence of a typical Type I strain. For ROP18, the Type III allele is the outlier, by far the most divergent and with only minimal expression level, while Type II and type I confer virulence on avirulent strains, thus not correlated with the IRG-related effects. Although the *T. gondii* susceptibility of mice deficient in single members of the IRG system guarantees that IRG proteins are major players in *T. gondii* resistance, it is certainly still possible that the IRG-related effects associated with the Type I clonal lineage are not functionally associated with any important parameter of virulence. It is, however, already clear that *T. gondii* virulence is determined by epistatic

interactions between alleles at multiple loci [27] so the single-locus gain-and-loss case analysed here may fail to reproduce the complexity of the natural situation.

T. gondii is not the only organism where virulence differences are associated with the mouse IRG system. As recently shown, the human pathogen, *Chlamydia trachomatis,* is resisted by interferon-induced mouse cells through the action of at least 3 IRG proteins, Irgm1, Irgm3 and Irgb10 [36,37,38] while the mouse-specific race, *C. muridarum* is not controlled. It has now been shown that the control of *C. trachomatis* and lack of control of *C. muridarum* is correlated with the presence or absence of IRG proteins, specifically Irgb10, on the *Chlamydia* inclusion [38]. The apparent dominance of the virulence in mixed infections is probably due to the presence of mixed inclusions (J. Coers, personal communication). C. *muridarum* and *C. trachomatis* are over 90% identical at the nucleotide level and share over 99% of their open reading frames [39,40], while virulent type I and avirulent types II and III *T. gondii* clonal lineages are greater than 95% identical at the nucleotide level [41], thus facilitating in both cases the search for loci responsible for these large differences in virulence. It is also likely that when the resistance mechanisms of the virulent parasite strains are revealed they will contribute to the elucidation of the IRG resistance mechanism itself.

The loading of IRG proteins onto vacuolar inclusions analysed here has been documented only for 2 intracellular organisms, *T. gondii* and *C. trachomatis*, while IRG-dependent resistance in mice has been documented for many other protozoal and bacterial pathogens [4]. In all other cases except *T. gondii* and *C. trachomatis*, however, evidence for the role of IRG proteins in these resistances derives exclusively from mice deficient in Irgm1, the only IRG protein so far which does not associate with the parasitophorous vacuole. The interpretation of these phenomena is much complicated by evidence that Irgm1 deficiency is associated with haemopoietic stem cell failure [42] and defective proliferative potential of T lymphocytes in animals infected with highly immunogenic parasites [43,44]. While a full explanation for this complex phenotype is still unknown, it has been shown recently that the lymphomyeloid deficiencies are corrected by interference with the IFNγ signalling pathway [45], suggesting that other IFNγ-inducible gene products are responsible for the lesion in the Irgm-deficient mouse. Recent results have implicated Irgm3, since the immunological defects (documented for *Salmonella typhimurium* resistance) are essentially reverted in Irgm1/Irgm3 double-deficient mice [46]. These results suggest strongly that susceptibilities documented exclusively in Irgm1-deficient animals result rather from an imbalance in the IRG system with broad effects on the

lymphomyeloid system, than from a cell-autonomous action in the infected cell [23]. Resistance to *T. gondii* does not fall into this paradigm: the Irgm1/Irgm3 doubly-deficient mouse is highly susceptible to the pathogen [46], and Irgm1/Irgm3 doubly-deficient IFNγ-induced MEFs are substantially less competent to control *T. gondii* replication than cells carrying either deficiency alone (unpublished results with Gregory A. Taylor). There is every reason to believe that the same applies to resistance to *C. trachomatis*. Thus we are led to the striking conclusion that the resistance mechanism associated with loading of multiple IRG proteins onto pathogen-containing vacuoles seen in *T. gondii*, *C. trachomatis* and probably also *L. pneumophila* infections (Joern Coers, personal communication) is of a distinctive class that truly reflects the cell-autonomous function of IRG proteins. Loss of resistance to *Salmonella*, *Mycobacteria*, *Trypanosoma*, *Listeria* etc in Irgm1-deficient mice reflects a non-cell-autonomous lesion due to the cytopathic consequences of unregulated IRG protein activation in interferon-induced cells of the lymphomyeloid system. An issue of considerable interest is therefore what the *T. gondii*, *C. trachomatis* and *L. pneumophila* vacuoles have in common to render them targets for IRG protein action, that is not shared with many other intracellular pathogens.

Materials and Methods.

Expression constructs

The following mammalian expression constructs were used: pEGFP-N3-Irga6-ctag1, pmCherry-N3 (both from [9]); pGW1H-Irgm1 [15]; pGW1H-Irgb6-FLAG, pGW1H-Irgm2, pGW1H-Irgm3 and pGW1H-Irgd (all from [16]). pmDsRed-N3-Irgb6-FLAG was made via PCR amplification of Irgb6-FLAG from pGW1H-Irgb6-FLAG construct using the following primers: forward 5'-ccccccccgtcgaccaccatggcttgggcctccagc-3' and reverse 5'-ccccccccgtcgaccttgtcatcgtcgtccttgtaatc-3' and insertion into pmDsRed-N3 (generous gift from Dr.Roger Y. Tsien, UCSD) following SalI digestion. pEGFP-N3-Irgb6-FLAG was generated by subcloning Irgb6-FLAG fragment from pmDsRed-N3-Irgb6-FLAG into pEGFP-N3 (Clontech) using SalI digestion. pmCherry-N3-Irgd-ctag1 was generated by PCR amplification of Irgd-ctag1 from pGW1H-Irgd-ctag1 [16] using the following primers: forward 5'-ccccccgtcgaccaccatggatcagttcatctcagcc-3' and reverse 5'-ccccccgtcgacgtcacgatgcggccgctcgagtcgg-3' and by cloning it into pmCherry-N3. pGW1H-unRop18 ME49 and pGW1H-unRop18 RH-YFP containing the unprocessed forms of Rop18 were generated by PCR amplification from genomic DNA of ME49 and RH-YFP *T. gondii* strains using the following primers: forward 5'-ccccccgtcgaccaccatgttttcggtacagcggcc -3' and reverse 5'-ccccccgtcgacttagtcaagtggatcctggttagtatggacctcttctgtgtggagatgttcctgc-3' and subsequent cloning into the SalI site of pGW1H. The C-terminally Ty-tagged mature forms of Rop18 were amplified from pGW1H-unRop18 ME49 and pGW1H-unRop18 RH-YFP using the following primers: forward 5'-ccccccgtcgaccaccatggaaagggctcaacaccgggta-3' and reverse 5'-ccccccgtcgacttagtcaagtggatcctggttagtatggacctcttctgtgtggagatgttcctgc-3' and cloned into pGW1H (British Biotech) by SalI digestion. Pfu-polymerase (Promega) was used for PCR amplification and primers were from Operon Biotechnologies GmbH. Restriction enzymes were from New England Biolabs. All constructs were verified by sequencing.

Cell culture

gs3T3 cells (Invitrogen), Mifepristone inducible gs3T3-Irga6 cells [16], A31 3T3 cells (ATCC, CCL-163), atg5$^{-/-}$ and the corresponding wt control immortalized mouse fibroblasts ([47], kindly provided by Martin Krönke and Nobura Mizushima), MyD88$^{-/-}$ MEFs ([48], kindly provided by Manolis Pasparakis), L929 cells (ATCC CCL-1), HFF cells (ATCC, CRL-1634) and C57BL/6 MEFs were cultured in DMEM, high glucose (Invitrogen) supplemented with 10% FCS

(Biochrom), 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM non-essential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin (all PAA). Transient transfection of mouse fibroblasts was conducted using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were induced with 200 U/ml of mouse IFNγ (Peprotech) or 10^{-9} M Mifepristone (Invitrogen) for 24 h.

To analyse the influence of other IRG proteins on the loading of Irga6 onto PVs of *T. gondii* strain ME49 pools of pGW1H-IRG expression constructs were transiently transfected into Mifepristone induced gs3T3-Irga6 cells [16] grown on cover slips in 6-well plates. In all cases 400 ng of each construct were cotransfected in a total amount 2 μg DNA. Empty vector was used to adjust DNA amounts if less than 5 constructs were transfected.

Immunological reagents

The following immunoreagents were used: rabbit anti-Irga6 antiserum 165/3 [15], mouse anti-Irga6 monoclonal antibodies 10E7 and 10D7 [21], mouse anti-Irgm3 monoclonal antibody anti-IGTP (BD Biosciences, 610881), goat anti-Irgb6 antiserum A20 (Santa Cruz Biotechnology, sc-11079), rabbit anti-Irgb6 141/1 was raised against bacterially synthesized full-length protein (unpublished data), mouse anti-Irgb6 monoclonal antibody B34 [24], rabbit anti-Irgm2 antiserum H53 [6], rabbit anti-Irgd antiserum 2078/3 [15], rabbit anti-Irgd 081/1 was raised against bacterially synthesized full-length protein (unpublished data), rabbit anti-Irgb10 antiserum [38], anti-Irgm1 antiserum L115 B0 [6], anti-*T. gondii* rabbit (BioGenex, PU125-UPE) and goat antisera (Abcam, ab23507), mouse IgG anti-GRA7 monoclonal antibody [20] and mouse IgM anti-GRA7 monoclonal antibody TxE2 ([49], rabbit anti-ctag1 antiserum 2600 [6], rabbit anti-calnexin antiserum (StressGene, SPA-865), mouse anti-α-tubulin monoclonal antibody (Sigma-Aldrich, T 6074), mouse anti-Ty tag monoclonal antibody ([50], kindly provided by Keith Gull), rat anti-LAMP1 monoclonal antibody (University of Iowa) anti-Akt (Cell Signaling, 9272) and anti-phosphoAkt (Cell Signaling, 9271) rabbit polyclonal antibodies, rabbit anti-PARP1 polyclonal antibody (Cell Signaling, 9542), Alexa 350/488/546/555/647 labelled donkey anti-mouse, -rabbit and -goat sera (Molecular Probes), donkey anti-rabbit- (GE Healthcare), donkey anti-goat- (Santa Cruz Biotechnology) and goat anti-mouse-HRP (horseradish peroxidase) (Pierce) antisera.

The serum 081/1 used to identify Irgd produced occasional signals in IFNγ-stimulated $Irgd^{-/-}$ MEFs. These signals were extremely faint compared to the strong signals from wild type cells

measured both by Western blot and immunofluorescence (unpublished data). Considering the significant homology between the IRG proteins we assume that the reagent is cross-reacting weakly on another family member. Nevertheless the counted frequency of positive vacuoles found with 81/1 corresponded with that seen with A20, a much weaker anti-peptide antiserum, used at high concentration.

Inhibition of signaling pathways and microtubule polymerization

To block PI3 kinase and G protein coupled receptors overnight FCS-starved C57BL/6 MEFs were pretreated with Wortmannin (0.5 μM), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) (25 μM) and Pertussis toxin (200 ng/ml) for 6 hours (all reagents were derived from Sigma-Aldrich and handled according to the manufacturers protocol). The extent of inhibition was tested by monitoring the level of pAkt after 10 min stimulation with EGF (epidermal growth factor) (100 ng/ml) (Peprotech) in Western Blot. To block caspase activity MEFs were pretreated with Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone) pan-caspase inhibitor (100 μM) (Alexis Biochemicals, 260-020-M005) for 2 h and the degree of blockade was analyzed by monitoring the processing of PARP1 (poly-ADP ribose polymerase 1) 6 h after TNFα (40 ng/ml) (Peprotech) plus Cycloheximide (Chx) (10 μg/ml) stimulation in Western blot. Inhibition of microtubule polymerisation was achieved by incubating MEFs in 10 μM nocodazole in DMSO (Sigma-Aldrich) for 1 h and monitored microscopically after performing immunostaining using anti-α-tubulin antibody.

To assay IRG protein association with the PV under conditions of blocked signalling pathways or inhibited microtubule polymerisation, IFNγ-stimulated MEFs were pretreated as described above or left untreated as a control, then infected with ME49 *T. gondii* for 2h and stained for Irgb6 (serum A20) and Irga6 (mAb 10D7). To study MyD88 involvement in PV loading by IRG proteins IFNγ-stimulated MyD88$^{-/-}$ and wt MEFs were infected with ME49 *T. gondii* and stained for Irgb6 (serum A20) and Irga6 (mAb10D7).

Passaging of *T. gondii* and infection of murine fibroblasts

The following *T. gondii* strains were used: type I virulent RH [51], RH ΔRop16 (Y-C Ong and J Boothroyd, unpublished), RH-YFP [52] and BK [53]; type II avirulent ME49 [54], NTE [55], avirulent recombinant *T. gondii* strains S22 [27] and S22 LC37 (J Boothroyd unpublished), the latter harbouring a cosmid expressing Rop5 genes from the RH genome. Tachyzoites of different

T. gondii strains were passaged in HS27 cells and used for infection of untreated, transiently transfected, IFNγ- and/or Mifepristone induced fibroblasts at a multiplicity of infection from 5 to 10 as described in Martens et al 2005. Infection was synchronized by centrifugation at 1500 rpm for 2 min and further performed for 2h or an otherwise specified period of time without washing off free parasites. When indicated free parasites were removed after an initial infection period of 15 min by repeated washing with PBS until no free parasites could be detected microscopically. At the end of infection free parasites were washed away by PBS and fixed in PBS/3% paraformaldehyde.

T. gondii proliferation assay

The growth of *T. gondii* was analysed using the ^{3}H-uracil incorporation assay [56]. IFNγ-induced MEFs were infected with specified *T. gondii* strains for 24h at MOI 0.3, 1 and 3. The cultures were further labelled with 0.3 μCi/well of ^{3}H-uracil (Harmann Analytic) for 24h and then frozen at -20°C. The amount of radioactivity incorporated into the proliferating parasites was determined by β-counter (number of counts per 3 minutes). The data is shown either directly in radioactivity counts (Fig S7) which is proportional to the parasite growth, or as the % parasite growth inhibition (Fig 9C) defined as proliferation of *T. gondii* in the presence of IFNγ (10 or 100 U/ml) divided by the proliferation in the absence of the cytokine (all background subtracted), the resulting value subtracted from 100%.

Immunocytochemistry

Immunocytochemistry was performed on paraformaldehyde fixed cells as described earlier [6], the images were taken with an Axioplan II fluorescence microscope and AxioCam MRm camera and processed by Axiovision 4.6 (now 4.7) (all Zeiss) and Image J software (http://rsb.info.nih.gov/ij/). 4′,6-Diamidine-2′-phenylindole dinydrochloride (DAPI, Invitrogen) was used for nuclear counterstaining at a final concentration of 0.5 μg/ml. Intracellular parasites were identified by observing the vacuolar localisation of the *T. gondii* protein GRA7 or by distinct pathogen appearance in phase contrast.

Live cell imaging

Live cells imaging was performed as described earlier [9].

Quantification of IRG protein signal intensity at *T. gondii* PV

The measurements were performed using the Image J (http://rsb.info.nih.gov/ij/) and Axiovision 4.7 (Zeiss) software. Two lines across each vacuole were drawn (Fig S1A) to define the pixel intensity profiles as shown on Fig S1B. The average intensity of the background area around each peak was subtracted from the average intensity of 4 peaks yielding a value of IRG protein mean pixel intensity around a vacuole.

SDS-PAGE and Western blot

Cells were lysed in 1% Triton X100/PBS/Complete Mini Protease Inhibitor Cocktail, EDTA free (Roche) or 1x Sample Buffer (80 mM Tris-HCI, 5 mM EDTA, 34% Sucrose, 3.2% SDS, 40 mM DTT, bromophenol blue (concentration)). Postnuclear supernatants were subjected to SDS-PAGE and Western blot. Membranes were probed for IRG proteins with the indicated primary and HRP-coupled secondary antibodies for chemiluminescence.

Figure legends

Figure 1. Time-course of Irga6+Irgb6 association with *T. gondii* ME49 PVs.

IFNγ-induced C57BL/6 MEFs were infected with *T. gondii* ME49 strain as described in materials and methods. At intervals from 2.5 minutes to 2 h after infection slides were prepared for staining simultaneously with antibody reagents against Irga6 (10D7) and Irgb6 (A20) using secondary antibodies coupled with the same fluorochrome to enhance the visible signal. DAPI was used to stain the nuclei. **(A)** Loading of IRG proteins begins early after cell penetration. Vacuoles with visible accumulations of IRG proteins on the PVM were counted per 1000 nuclei at each time point. One representative experiment out of 2 independent repetitions is shown. **(B)** IRG signal intensity at the PVM increases with time after infection. Fluorescent IRG protein (Irgb6 and Irga6) signal intensities on individual randomly chosen vacuoles were measured as described in materials and methods at the times indicated. Neither signal intensities nor heterogeneity were detectably affected by removal of free parasites. Open circles: free parasites were washed off 15 minutes after inoculation; closed circles: free parasites were not washed off after inoculation. 20 positive vacuoles were measured at each time point. One representative experiment out of 2 independent repetitions is shown.

Figure 2. Loading of individual vacuoles by transfected Irga6-ctag1-EGFP or Irgb6-FLAG-EGFP observed by time-lapse microscopy.

C57BL/6 MEFs were transfected with the expression plasmid pEGFP-Irga6-ctag1-EGFP or pEGFP-Irgb6-FLAG-EGFP and simultaneously induced with IFNγ. After 24 h, the cells were infected with *T. gondii* ME49 strain in a microscope slide chamber (materials and methods). Cells were observed continuously to document the entry of individual parasites and the subsequent accumulation of Irga6-ctag1-EGFP or Irgb6-FLAG-EGFP on the PV. (**A** and **B**) Selected frames of two time-lapse videos of Irga6-ctag1-EGFP loading on ME49 *T. gondii* PV. Arrowheads indicate the location of the analysed *T. gondii* PVs. The arrow on Fig 2B indicates a *T. gondii* vacuole already loaded with Irga6-ctag1-EGFP before the initiation of the movie (see also text). (Note that the frames shown in Fig 2B are not a regular time series since some frames were out of focus and have not been included). The videos from which frames on Fig 2A and Fig 2B were extracted are presented as video S1 and video S2 respectively. **(C)** Mean pixel intensities of Irga6 and Irgb6 at the PVM were measured from the vacuoles shown in Fig 2A and Fig 2B (Irga6 I and Irga6 II respectively) and from 2 further videos of Irgb6 (Irgb6 I and Irgb6 II,

movies not shown), and plotted as percentage of the maximum intensity. The origin on the time axis is the time of addition of *T. gondii* to the cells. The first symbol of each plot gives the time when the observed parasite was seen to enter the cell. In case of Irgb6 I movie the protein signal slightly decreases after 13 min due to focus drift on 15 and 17 min frames and it resumes to rise after correction.

Figure 3. Influence of duration of IFNγ-induction of Irga6 and Irgb6 protein levels on vacuolar loading.

MEFs were induced for different times with IFNγ before infection with *T. gondii* strain ME49 for 2 h and stained in immunofluorescence against Irga6 and Irgb6. Co-staining against GRA7 was used to determine intracellular parasites. The pixel intensities of **(A)** Irga6 (165) and **(B)** Irgb6 (A20) signals at the PVM of ME49 vacuoles were determined as described in materials and methods (see also Fig S1) and displayed as a function of IFNγ-induction time. 60 PVs were quantified per time point and the arithmetic means are given as horizontal lines. **(C)** In parallel sample cell lysates from MEFs induced for the indicated times with IFNγ were analysed by Western blot for Irga6 (10D7) and Irgb6 (B34) expression level relative to calnexin as a loading control.

Figure 4. Vacuolar loading of IRG proteins is independent of major signalling systems and microtubules.

C57BL/6 MEFs were induced with IFNγ and treated as described in materials and methods with inhibitors of PI3-kinase (wortmannin and LY294002), G-protein-coupled receptors (pertussis toxin), caspases (z-VAD-fmk) and microtubule polymerisation (nocodazole). Multiple TLR-mediated signals were excluded in IFNγ-induced MEFs from MyD88-deficient mice (see materials and methods). The efficacy of each treatment was assayed as described in materials and methods and as shown in Fig S2. Untreated, treated and MyD88-deficient cells were infected with *T. gondii* ME49 strain for 2 hours and stained separately with antibody reagents against Irga6 (10D7) and Irgb6 (A20). The frequency of vacuoles detectably positive for Irga6 and Irgb6 was calculated as a percentage from 200-400 intracellular parasites. One representative experiment out of 2 independent repetitions is shown.

Figure 5. Atg5 regulates loading of IRG proteins onto *T.gondii* PV.

(A) IRG protein association with *T. gondii* ME49 PVs is reduced in atg5$^{-/-}$ fibroblasts. Wt and atg5$^{-/-}$ fibroblasts were induced for 24 hours with IFNγ and infected with *T. gondii* ME49 strain for 2h. Irga6, Irgb6 and Irgd positive vacuoles were detected by staining with antibodies 10D7, A20 and 081/1 respectively. 100-200 intracellular parasites per experiment were counted and the mean values and standard deviations of 2 experiments are presented. **(B)** The intensity of Irgb6 and Irga6 vacuolar loading is reduced in atg5$^{-/-}$ cells. Loading intensity was measured as described in materials and methods on at least 40 vacuoles from the experiment shown in Fig 5A. Horizontal bars represent the arithmetic mean values. **(C)** Irga6, Irgb6, Irgd and Irgm2 levels are reduced in atg5$^{-/-}$ MEFs while Irgm1 and Irgm3 are unaffected. Wt and atg5$^{-/-}$ fibroblasts were induced with IFNγ for 24h and analysed by Western blot with antibody reagents detecting the following IRG proteins: Irga6 (10D7), Irgb6 (B34), Irgd (2078), Irgm2 (H53), Irgm1 (L115) and Irgm3 (anti-IGTP). **(D)** IRG proteins form aggregates in IFNγ-induced atg5$^{-/-}$ MEFs. Cells were induced with IFNγ, infected with *T. gondii* ME49 strain and prepared for microscopical analysis as described in Fig 5A. Rabbit anti-*Toxoplasma* serum (upper and middle panels) or anti-GRA7 (lower panel) monoclonal antibody was used to identify the pathogen. Arrows indicate intracellular parasites. Phase: phase contrast. The arrowheads indicate the IRG protein aggregates.

Figure 6. IRG proteins load in a consistent hierarchy on to the PV of *T. gondii* ME49 strain.

(A) Each IRG protein loads onto a characteristic proportion of vacuoles. Quantification of IRG positive PVs (%) observed in IFNγ-induced *T. gondii* ME49 infected MEFs and gs3T3 cells assayed by immunocytochemistry using antibody reagents described in Fig. S4 and materials and methods. At least 2 independent experiments were assayed and pooled and a minimum of 500 PVs counted for each IRG protein. **(B)** IRG proteins do not load at random onto each vacuole. IRG proteins loaded onto *T. gondii* PV were detected by co-staining with pairs of specific antibodies and specific secondary reagents carrying different fluorochromes. Vacuoles loaded with one IRG protein were scored for possession of the second and vice versa. Vacuoles loaded with neither IRG protein were not included in the analysis. At least 100 positive vacuoles were counted for each pair of IRG proteins. Their approximately frequencies can be estimated from the data in Fig 6A. The PV loading with IRG proteins is very strongly correlated. The full data are shown in table S1. **(C)** Irgb6 loads more heavily onto *T. gondii* vacuoles at early time points then

Irga6. C57BL/6 MEFs were induced with IFNγ and infected with *T.gondii* ME49 strain. At indicated times after infection Irgb6 and Irga6 vacuole loading intensities were analysed simultaneously with specific primary antibodies (Irgb6, A20; Irga6, 10D7) detected with secondary antibodies labeled with different fluorochromes. **(D)** Irgb6 loads before Irgd on to the *T. gondii* ME49 strain PV. C57BL/6 MEFs were induced with IFNγ and transfected simultaneously with constructs expressing Irgb6-FLAG-EGFP and Irgd-ctag1-Cherry. After 24 h, cells were infected with *T. gondii* ME49 strain in microscope slide chambers and observed by live-cell imaging for the accumulation of IRG proteins. Successive one-minute frames from one vacuole show Irgb6-FLAG-EGFP visibly loading several minutes before Irgd-ctag1-Cherry. See also Fig. S4. **(E)** Absence of Irga6 does not affect the proportion of vacuoles loaded with Irgb6 or Irgd. Irga6$^{-/-}$ and wild type MEFs were induced with IFNγ and infected with *T. gondii* strain ME49. 2 h after infection cells were stained with appropriate antibody reagents and the proportion of Irgb6 (B34) and Irgd (081/1) labelled vacuoles (out of 300 for each IRG protein) was recorded. **(F)** Intensity of Irgb6 PV loading of Irgb6 is significantly reduced in Irga6-/- MEFs. IFNγ-induced Irga6$^{-/-}$ and wild type MEFs were infected with *T. gondii* ME49 strain. 2 h after infection slides were stained for Irgb6 (B34), Irgd (081/1) and Irgm2 (H53). At least 50 vacuoles loaded with each IRG protein were assayed for loading intensity from both cell types.

Figure 7. Loading of Irga6 at the *T. gondii* ME49 strain PV is enhanced by the presence of other IRG proteins of the GKS group.

gs3T3-Irga6 cells were induced with IFNγ or Mifepristone. At the same time, Mifepristone-induced cells were transfected with pools of constructs (see materials and methods for experimental details) expressing either the three GMS proteins, (Irgm1, Irgm2 and Irgm3) alone to permit access of Irga6 to the PV (3GMS) or, in addition to the 3GMS proteins, also Irgb6 (3GMS+b6), Irgd (3GMS+d) or both Irgb6 and Irgd (5IRGs). After 24 h, cells were infected with *T. gondii* ME49 strain for 2 h. Irga6 was detected at the PV in transfected cells using antibody 10E7 in immunofluorescence. Transfected cells were identified by staining for Irgm2 with the H53 serum. Vacuolar loading of Irga6 was enhanced by addition of Irgb6 or Irgd.

Figure 8. Accumulation of IRG proteins on the PVM is reduced in virulent *T. gondii* infection.

(A) IFNγ-induced MEFs were infected for 2 h with type II avirulent (ME49 and NTE) and type I virulent (RH and BK) *T. gondii* strains and assayed microscopically for Irgb6-positive vacuoles (serum A20). Irgb6 positive PVs are shown as percentage among 400-500 intracellular parasites. **(B)** gs3T3 cells were induced with IFNγ and infected with *T. gondii* ME49 strain (black bars) or RH-YFP strain (white bars). Numbers of Irgb6, Irgb10, Irga6 and Irgd positive PVs were counted in 2-6 experiments for each IRG protein and *T. gondii* strain and given as a percentage of intracellular parasites. More than 200 intracellular parasites were counted blind per experiment. **(C)** C57BL/6 MEFs were induced with IFNγ and infected with RH-YFP. Irgb6 (blue) and Irga6 (red) were detected in immunofluorescence with the A20 serum and the 10E7 monoclonal antibody respectively. Intracellular fluorescent parasites (RH-YFP, green) identified in phase contrast (PhC) are indicated by white arrowheads (strongly IRG positive) and arrows (weakly IRG positive). **(D)** gs3T3 fibroblasts were induced with IFNγ and infected with either ME49 or RH-YFP *T. gondii* strains. Mean fluorescence intensities of Irga6 (165) and Irgb6 (A20) signals at the PVM were quantified as described in Fig S1 and materials and methods. 35 random PVs per data set were quantified blind. **(E)** C57BL/6 MEFs were induced with IFNγ and infected with *T. gondii* RH-YFP strain. Mean fluorescence intensities of Irga6 and Irgb6 were measured for groups of PVs expressing no detectable (open circles), weak Irga6 (grey filled circles) or strong Irga6 staining (black filled circles). The fluorescent intensity profiles of 5 representative PVs per group are displayed in supplementary Fig S5. **(F)** Photomicrograph of a doubly infected for 2h IFNγ-stimulated MEF shows an ME49 strain *T. gondii* (indicated by arrow) with intense Irgb6 (A20) accumulation at the PV (red) while a green RH-YFP (indicated by arrowhead) in the same cell has no Irgb6 on the PV. **(G)** IFNγ stimulated MEFs were infected with *T. gondii* Me49 strain alone or simultaneously with ME49 and RH-YFP strains. Irgb6 (detected by A20) and Irga6 (detected by 10D7) fluorescence intensities were measured on at least 30 ME49 PVs in singly and doubly infected cells. Me49 and RH-YFP were discriminated by the fluorescent signal from RH-YFP. The loading of Irga6 and Irgb6 onto PV of avirulent ME49 strain *T. gondii* was unaffected by the simultaneous presence of virulent RH-YFP.

Figure 9. Rop18, Rop16 and Rop5 virulence-associated *T. gondii* kinases do not affect IRG-mediated control of the parasite.

(A) Ectopically expressed Rop18 does not affect loading of *T. gondii* ME49 PV with Irgb6 in infected L929 cells. L929 cells were induced with IFNγ, transfected with pGW1H expression

plasmids encoding the mature form of ROP18 from either ME49 or RH-YFP *T. gondii* strains, or pmCherry as a transfection control and infected for 2 h with *T. gondii* ME49 strain. Cells were stained for Irgb6 with A20 serum and for Ty-tag to identify the transfected cells. Irgb6-positive vacuoles in Rop18-Ty-tag and Cherry positive cells were enumerated. **(B)** Rop16 and Rop5 kinases do not affect Irgb6 loading onto *T. gondii* PV. IFNγ stimulated MEFs were infected with the S22 LC37 *T. gondii* strain expressing the Rop5 gene cluster (see also materials and methods and main text), RH ΔRop16 and control parental strains S22 and RH for 2h. Irgb6 (stained with 141/1 serum) positive PVs were quantified from 200-400 intracellular parasites. **(C)** The IFNγ-mediated growth inhibition of S22 LC37 and RH ΔRop16 *T. gondii* strains is comparable to the inhibition of S22 and RH control strains in MEFs. Proliferation of *T. gondii* strains was measured by ^{3}H-uracil incorporation and presented as a percentage of *T. gondii* growth inhibition as described in materials and methods. Black and grey bars represent the extent of parasite growth control at 10 and 100 U/ml of IFNγ cell stimulation respectively. See Fig S7 for untransformed data.

Supporting information

Figure S1. Analysis of IRG pixel intensity on *T. gondii* PVs.

The details of analysis are given in materials and methods. To measure the intensity of IRG signal on *T. gondii* PVs two lines across each vacuole were drawn **(A)** to define the pixel intensity profiles **(B)** across these lines using the Image J and Axiovision 4.7 software. To investigate how much variation has arisen from the inhomogeneouty of IRG signal on PVs 40 profiles for 3 morphologically different Irgb6 positive vacuoles (stained by A20) were created by drawing the corresponding number of lines through the PVs. The mean values of pixel intensity were calculated from each profile and presented as a single dot on **(C)**. In this case variation of Irgb6 intensity on PVs produced by the mean values of 2 points across each PV does not account for the total heterogeneity of IRG protein signal shown on Fig 2**(A and B).** Moreover variation in IRG protein intensity could be reduced using 4 points on each vacuole.

Figure S2. Inhibition of major signalling pathways and microtubule polymerisation.

(A) The blockade of PI3 kinase and G protein coupled receptors was demonstrated by Western blot for phospho-Akt (pAkt). MEFs were treated with wortmannin (W), LY294002 (LY) or

pertussis toxin (PT) for 6h as described in materials and methods. Inhibited cells were stimulated by EGF for 10 min to induce Akt phosphorylation. Calnexin served as a loading control. **(B)** To inhibit caspases MEFs were treated with z-VAD-fmk for 2h followed by stimulation with TNFα for 6 h in the presence of cycloheximide, as described in materials and methods. The amount of PARP1 processing by caspases was analysed by Western Blot. **(C)** Inhibition of microtubule polymerisation was monitored microscopically by immunostaining with an anti-tubulin antibody. MEFs were induced with IFNγ for 24 h and treated with nocodazole, or DMSO as control, for 1 hour, as described in materials and methods. Treated cells were infected with *T. gondii* ME49 strain and loading of parasite PVs was monitored 2 h after infection by immunostaining for Irgb6 (A20).

Figure S3. Irga6 aggregates do not colocalise with lysosomal marker LAMP1 in atg5$^{-/-}$ fibroblasts. Cells were prepared as described in Figure 5A and immunostained for Irga6 (10D7) and LAMP1 (1D4B). No obvious collocalisation of Irga6 with LAMP1 in wt and atg5$^{-/-}$ fibroblasts was observed. Arrows indicate intracellular parasites identified by phase contrast and arrowheads indicate Irga6 aggregates.

Figure S4. Frequencies of IRG protein-positive vacuoles detected by specific antibody reagents at different dilutions: saturation of frequency estimates.
IFNγ-induced C57BL/6 MEFs were infected with *T. gondii* ME49 for 2h. IRG proteins were detected by serial two-fold dilution of antibody reagents around the dilutions used experimentally followed by an appropriate secondary reagent to estimate loaded vacuole frequencies. The experimental dilutions for each reagent were as follows: for Irgb6: B34 at 1/1000 (3.4 mg/ml stock), A20 at 1/200; for Irgb10: anti-Irgb10 at 1/2000; for Irga6: 10E7 at 1/1000 (3.7 mg/ml stock), 10D7 at 1/2000 (12.56 mg/ml stock), 165 at 1/8000; for Irgd, 081/1 at 1/4000. The most concentrated dilution is designated as 1 (B34 at 1:250, A20 at 1:50, anti-Irgb10 at 1:500, 10E7 at 1:100, 10D7 at 1:500, 165 at 1:2000, 081/1 at 1:4000). IRG positive PVs were counted blind from 100-200 intracellular parasites. Frequency estimates for loaded vacuoles were independent of antibody or antiserum dilution in the range tested.

Figure S5. Irga6-ctag1-EGFP loads before Irgd-ctag1-Cherry onto *T. gondii* ME49 strain PV.

C57BL/6 MEFs were induced with IFNγ and transfected simultaneously with constructs expressing Irga6-ctag1-EGFP and Irgd-ctag1-Cherry. After 24 h, cells were infected with *T. gondii* ME49 strain in microscope slide chambers and observed by live-cell imaging for the accumulation of IRG proteins. Successive 3-minute frames from one vacuole show Irga6 visibly loading several frames before Irgd.

Figure S6. Irga6 and Irgb6 pixel intensity profiles of RH-YFP PVs in IFNγ-induced cells.
IFNγ-induced MEFs were infected with the *T. gondii* strain RH-YFP for 2 h. The pixel intensities of the Irga6 (10D7, red) and Irgb6 (A20, blue) signals at the RH-YFP (green) PVM across transects of the vacuole are displayed as a function of distance (μm). 5 representative PVs of the 3 categories Irga6 and Irgb6 double positive **(A)**, Irga6 single positive **(B)** and double negative **(C)** are given (see also Fig. 8E).

Figure S7. INFγ-restrained proliferation of *T. gondii* is not affected by the virulence kinases Rop5 and Rop16.
The growth of RH ΔRop16, S22 LC37 (carrying the plasmid expressing 4 linked Rop5 genes) and control RH, S22 *T. gondii* strains was measured by ^{3}H-uracil incorporation as described in materials and methods. The data are presented in radioactive counts. Black, grey and brown bars indicate proliferation of the parasite in MEFs untreated, stimulated with 10 U/ml and 100 U/ml of IFNγ respectively.

Video S1. Time-lapse video of Irga6-ctag1-EGFP association with the *T. gondii* PV.
IFNγ treated MEFs were transfected with the construct expressing Irga6-ctag1-EGFP and infected with *T. gondii* ME49. Time-lapse video was started immediately after infection and the images were collected every 30 seconds. The video is presented at 6 frames per second.

Video S2. Time-lapse video of Irga6-ctag1-EGFP association with the *T. gondii* PV.
IFNγ treated MEFs were transfected with the construct expressing Irga6-ctag1-EGFP and infected with *T. gondii* ME49. Time-lapse video was started 1 hour after infection and the images were collected every 3 minutes. The video is presented at 2 frames per second. Out of focus frames were excluded from the movie.

Table S1. Summary of the analyses of individual IRG protein loading onto *T. gondii* ME49 PV in relation to the loading of the other family members.

	Only A	A+B	Only B	Total A+B
Irgb6 *vs* Irgb10	159	1142	130	1431
% of total PVs	11.1%	79.8%	9.1%	
Irgb6 *vs* Irga6	212	612	10	834
% of total PVs	25.4%	73.3%	1.2%	
Irgb6 *vs* Irgd	419	283	1	703
% of total PVs	59.55%	40.25%	0.14%	
Irgb6 *vs* Irgm2	298	310	1	609
% of total PVs	48.9%	50.9%	0.1%	
Irgb10 *vs* Irga6	91	216	11	318
% of total PVs	28.6%	67.9%	3.5%	
Irga6 *vs* Irgd	327	390	0	718
% of total PVs	45.5%	54.3%	0.14%	
Irga6 *vs* Irgm2	36	113	0	149
% of total PVs	24.2%	75.8%	0	
Irga6 *vs* Irgm3	10	101	0	111
% of total PVs	9.0%	91.0%	0	

IFNγ stimulated mouse fibroblasts were infected with *T. gondii* ME49 for 2h and doubly or triply co-stained for distinct IRG proteins using appropriate immunoreagents as described in Fig S4 and materials and methods. Vacuoles positive for any IRG protein were analysed for the presence of the other protein of the family and single and double IRG protein positive PVs were quantified. The data are presented with the first and second IRG protein designated as A and B respectively. (Only A), (Only B) and (A+B) indicate number of vacuoles positive for only first, only second or positive for both IRG proteins. At least 100 individual vacuoles were scored. Vacuoles loaded with neither IRG protein were not included in the analyses. See also Fig 6B.

Acknowledgements

We thank Rita Lange, Mattias Muellenbeck and Ashiq Hussain for valuable technical support at various points during this study, Daniel Zivkovic for assistance with statistical analysis and Nikolaus Pawlowski for purifying bacterially expressed Irgb6 and Irgd proteins. We are grateful to the following scientists for the provision of specific reagents, acknowledged individually in the text: Douglas Carlow, Keith Gull, Martin Kroenke, Nobura Mizushima, Manolis Pasparakis, Roger Y. Tsien and Jens Zerrahn.

AK, JPH and DP were supported in part by the International Graduate School for Genetics and Functional Genomics and by the Graduate School for Biological Sciences of the University of Cologne. This work was supported by the following grants to JCH from the Deutsche Forschungsgemeinschaft: SPP1110, Innate Immunity; SFB670, Cell Autonomous Immunity; SFB635 Post-translational Control of Protein Function; SFB680 Molecular Basis of Evolutionary Innovation.

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