



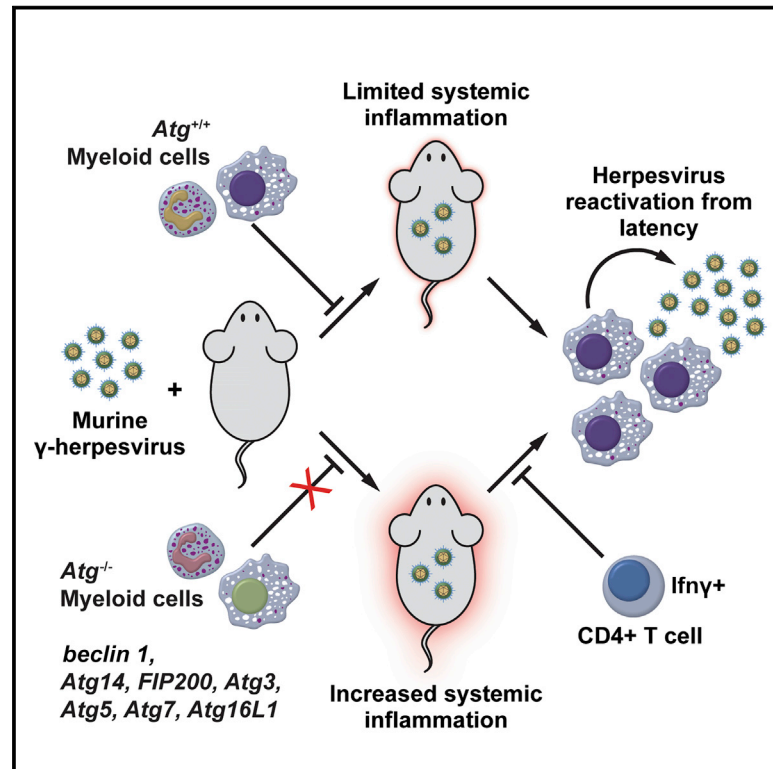
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# Cell Host & Microbe

## Autophagy Genes Enhance Murine Gammaherpesvirus 68 Reactivation from Latency by Preventing Virus-Induced Systemic Inflammation

### Graphical Abstract



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### In Brief

Park et al. report that canonical autophagy genes in myeloid cells regulate the level of systemic inflammation induced by chronic murine gammaherpesvirus infection. One consequence of increased systemic inflammation in the setting of autophagy-gene deficiency is the inhibition of reactivation of the herpesvirus from latently infected macrophages.

### Highlights

- Autophagy (*Atg*) genes in myeloid cells inhibit virus-triggered systemic inflammation
- *Atg* gene-regulated systemic inflammation inhibits herpesvirus reactivation
- Interferon- $\gamma$  controls herpesvirus reactivation in the setting of *Atg* gene mutations



# Autophagy Genes Enhance Murine Gammaherpesvirus 68 Reactivation from Latency by Preventing Virus-Induced Systemic Inflammation

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## SUMMARY

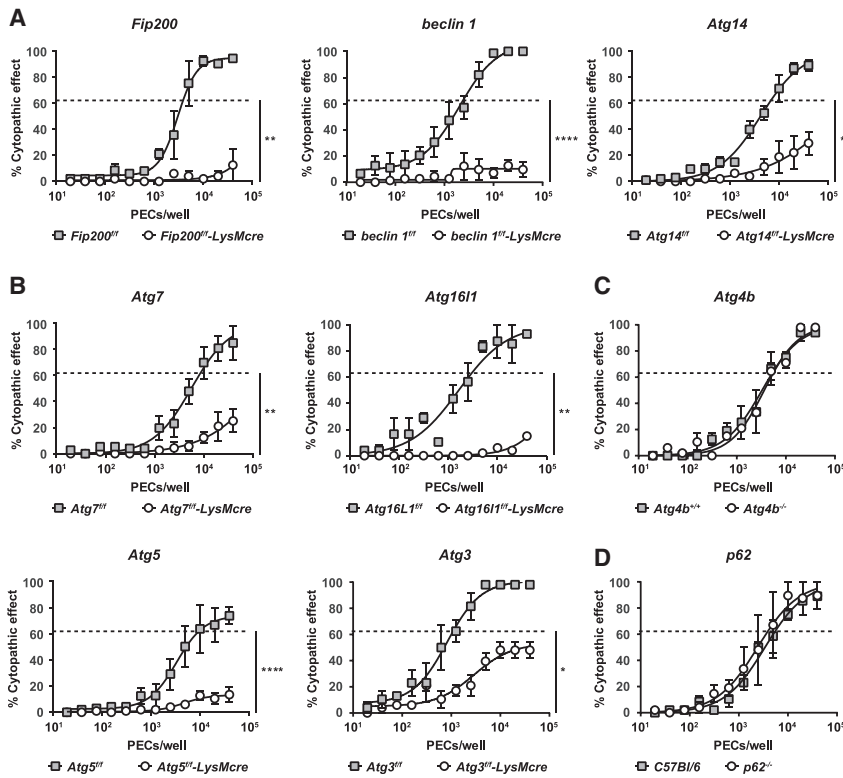
Host genes that regulate systemic inflammation upon chronic viral infection are incompletely understood. Murine gammaherpesvirus 68 (MHV68) infection is characterized by latency in macrophages, and reactivation is inhibited by interferon- $\gamma$  (IFN- $\gamma$ ). Using a lysozyme-M-cre (*LysMcre*) expression system, we show that deletion of autophagy-related (*Atg*) genes *Fip200*, *beclin 1*, *Atg14*, *Atg16l1*, *Atg7*, *Atg3*, and *Atg5*, in the myeloid compartment, inhibited MHV68 reactivation in macrophages. *Atg5* deficiency did not alter reactivation from B cells, and effects on reactivation from macrophages were not explained by alterations in productive viral replication or the establishment of latency. Rather, chronic MHV68 infection triggered increased systemic inflammation, increased T cell production of IFN- $\gamma$ , and an IFN- $\gamma$ -induced transcriptional signature in macrophages from *Atg* gene-deficient mice. The *Atg5*-related reactivation defect was partially reversed by neutralization of IFN- $\gamma$ . Thus *Atg* genes in myeloid cells dampen virus-induced systemic inflammation, creating an environment that fosters efficient MHV68 reactivation from latency.

## INTRODUCTION

Herpesvirus infections are life-long due to the establishment of latency, a molecularly quiescent form of infection, and reactivation from latency to generate infectious virus (Speck and Ganem,

2010). Chronic human gammaherpesvirus infection with Epstein-Barr virus or Kaposi's sarcoma-associated herpesvirus is associated with lymphoproliferative diseases (Cesarman et al., 1995; Chang et al., 1994; Nador et al., 1996; Young et al., 1989). Infection of mice with the related murine gammaherpesvirus 68 (MHV68,  $\gamma$ HV68, MuHV4) also results in lymphoproliferative disease (Tarakanova et al., 2005, 2008). After resolution of acute productive infection, MHV68 persists in mice by establishing latency in peritoneal macrophages and splenic B cells independent of the route of infection (Flaño et al., 2000; Tibbetts et al., 2003; Weck et al., 1996, 1999a, 1999b). Interferon- $\gamma$  (IFN- $\gamma$ ) inhibits, and IL-4 increases, reactivation from latently infected macrophages by regulating viral promoters for the essential immediate early *gene 50* (Barton et al., 2011; Goodwin et al., 2010; Reese et al., 2014; Steed et al., 2006, 2007).

Macroautophagy (termed canonical autophagy herein) degrades cytoplasmic cargo captured within double membrane-bound autophagosomes, which fuse with lysosomes to generate autolysosomes (Levine et al., 2011). We refer to canonical autophagy to distinguish it from topologically distinct cellular processes that require certain *Atg* genes such as LC3-associated phagocytosis (LAP), secretion, and control of parasite and viral replication by IFN- $\gamma$  (Bestebroer et al., 2013; Choi et al., 2014; DeSelm et al., 2011; Henault et al., 2012; Hwang et al., 2012; Martinez et al., 2015; Reggiori et al., 2010; Sanjuan et al., 2007; Zhao et al., 2008). Canonical autophagy involves activation of the ULK1 complex (ULK1-ATG13-FIP200-ATG101) and the class III phosphatidylinositol-3-OH kinase (PI3K) complex (VPS34-VPS14-Beclin 1-ATG14). Two ubiquitin-like protein conjugation systems conjugate LC3 family members to phosphatidylethanolamine and ATG12 to ATG5 (Weidberg et al., 2010, 2011) in reactions requiring ATG7 as an E1-like enzyme. ATG10, ATG5, and ATG16L1 are involved in generating ATG5-ATG12 conjugates while ATG3 and ATG4 are involved in LC3 lipidation. SNAREs



**Figure 1. Multiple Autophagy Genes Promote MHV68 Reactivation from Macrophages**

(A–D) *Fip200<sup>fl/fl</sup>-LysMcre*, *beclin 1<sup>fl/fl</sup>-LysMcre*, and *Atg14<sup>fl/fl</sup>-LysMcre* mice (A); *Atg7<sup>fl/fl</sup>-LysMcre*, *Atg161<sup>fl/fl</sup>-LysMcre*, *Atg5<sup>fl/fl</sup>-LysMcre*, and *Atg3<sup>fl/fl</sup>-LysMcre* mice (B); *Atg4b<sup>-/-</sup>* (C); and *p62<sup>-/-</sup>* (D) and wild-type littermates were infected with  $1 \times 10^6$  PFU MHV68 intraperitoneally (i.p.) for 42 days to determine frequency of virus reactivation in peritoneal exudate cells (PECs) during latent infection using LDA analysis. Data points indicate the percentage of wells that were positive for cytopathic effect (CPE) on a mouse embryonic fibroblast (MEF) monolayer at a given cell dilution. All LDA experiments are  $n = 3$  or 4 with 3–5 mice pooled per experiment. Data are the mean  $\pm$  SEM and  $p$  values were obtained by paired  $t$  test over all dilutions. Only significant comparisons are indicated. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.001$ .

## RESULTS

### Multiple Autophagy Genes Promote MHV68 Reactivation from Macrophages

To investigate whether *Atg* genes regulate chronic MHV68 infection, we used mice with *Atg* genes flanked by loxP sites,

mediate the fusion of autophagosomes and lysosomes (Diao et al., 2015; Itakura et al., 2012; Nair et al., 2011).

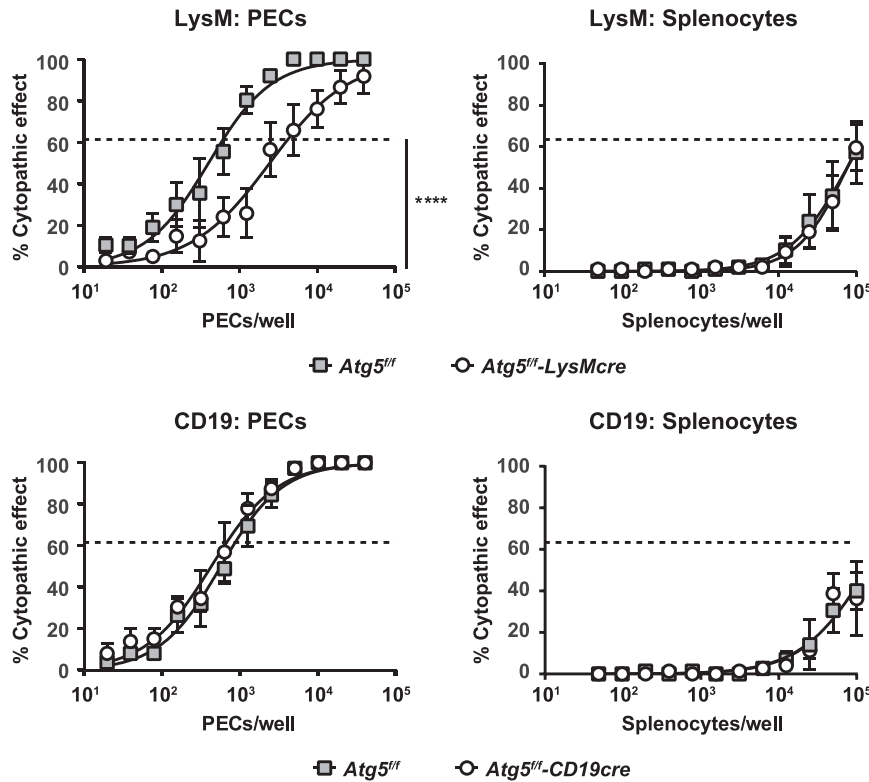
*Atg* genes regulate innate and adaptive immunity (Dupont et al., 2011; Lupfer et al., 2013; Nakahira et al., 2011; Saitoh et al., 2008; Shi et al., 2012) and participate in LAP (Henault et al., 2012; Huang et al., 2009; Martinez et al., 2011, 2015; Sanjuan et al., 2007). Autophagy and *Atg* gene-dependent cellular functions control intracellular pathogens such as viruses (Hwang et al., 2012; Orvedahl et al., 2007, 2010) and are countered by herpesvirus virulence proteins (E et al., 2009; Lee and Sugden, 2008; Lee et al., 2009; Leidal et al., 2012; Liang et al., 2013; Loh et al., 2005; Orvedahl et al., 2007; Takahashi et al., 2009; Yordy et al., 2012).

Since *Atg* genes and canonical autophagy control both viral replication in a cell-intrinsic manner and inflammatory processes that might influence viral infection in a cell-extrinsic manner, we defined the role of *Atg* genes during MHV68 infection. We found that myeloid cell expression of multiple *Atg* genes was required for efficient MHV68 reactivation from murine macrophages, but not for viral replication or establishment of latency. This function of *Atg* genes was through inhibition of virus-triggered systemic inflammation rather than effects intrinsic to infected cells. Some *Atg* genes required for efficient reactivation are not required for LAP, arguing that canonical autophagy inhibited virus-triggered systemic inflammation. Thus, *Atg* genes prevent excessive systemic inflammation during chronic herpesvirus infection. Interestingly, in a companion paper we found that certain *Atg* genes act in myeloid cells to prevent lung inflammation and thereby foster lethal influenza virus infection (Lu et al., 2016). Together these studies suggest that a common role for *Atg* genes in myeloid cells is to prevent tissue-specific and virus-induced inflammation and that this can have significant effects on infectious disease.

*Atg<sup>flox/flox</sup> (Atg<sup>fl/fl</sup>)*, bred to mice expressing Cre recombinase under the control of the lysozyme-M (*LysMcre*) promoter (Clausen et al., 1999; Hwang et al., 2012; Jakubzick et al., 2008). Several studies have demonstrated canonical autophagy-independent functions of *Atg* genes (Choi et al., 2014; DeSelm et al., 2011; Henault et al., 2012; Hwang et al., 2012; Sanjuan et al., 2007; Zhao et al., 2008). We selected *Atg* genes involved in various stages of autophagy, recognizing that in macrophages *beclin 1*, *Atg5*, *Atg7*, *Atg161*, and *Atg3* are required for both canonical autophagy and LAP, while *Fip200* and *Atg14* are not required for LAP (Martinez et al., 2015).

We assessed reactivation from latency 42 days after MHV68 infection, when productive infection has been cleared, using a limiting dilution reactivation assay (LDA) in which cell populations containing latently infected cells are explanted onto monolayers of murine embryonic fibroblasts (MEFs) for 2–3 weeks and the emergence of infectious virus is scored by detecting virus-induced cytopathic effects on the MEF monolayer (Weck et al., 1996). The presence of preformed infectious virus in samples was assayed in parallel by disrupting explanted cells in a manner that prevents reactivation but that does not inactivate infectious virus.

MHV68 reactivation from peritoneal exudate cells (PECs) is overwhelmingly from latently infected macrophages (Weck et al., 1999b). MHV68 reactivated inefficiently from peritoneal macrophages from mice lacking *Fip200*, *beclin 1*, or *Atg14* in LysM-expressing cells (Figure 1A). LysM-specific deletion of genes involved in the ubiquitin-like conjugations systems of autophagy including *Atg5*, *Atg7*, *Atg161*, and *Atg3* resulted in a similar defect in MHV68 reactivation (Figure 1B). The difference between mice mutated for *Atg3* versus other genes is



**Figure 2. Autophagy Genes Control MHV68 Reactivation from Macrophages, Not from B Cells**

*Atg5<sup>ff</sup>*, *Atg5<sup>ff</sup>-LysMcre*, and *Atg5<sup>ff</sup>-CD19cre* mice were infected with  $1 \times 10^6$  PFU MHV68 i.p. for 16 days, and LDA was performed on PECs and splenocytes to measure virus reactivation. Data are combined from three biological replicate experiments with 3–5 mice pooled per experiment. Data are the mean  $\pm$  SEM and p values were obtained by paired t test over all dilutions. Only significant comparisons are indicated. \*\*\*\*p < 0.001.

likely related to inefficient deletion of this gene in these lineages (Choi et al., 2014). Cells from *Atg4b<sup>-/-</sup>* mice reactivated normally (Figure 1C). There was no reactivation from splenocytes at this time point (Figure S1A and data not shown), and preformed infectious virus did not contribute to observed reactivation (Figure S1B). p62, an autophagy adaptor responsible for targeting Sindbis virus capsids to the autophagosome (Orvedahl et al., 2010), did not regulate MHV68 reactivation (Figure 1D). Thus, multiple *Atg* genes, but not p62, promote the MHV68 reactivation from latently infected macrophages. For subsequent studies, we focused on MHV68 reactivation using mice lacking *Atg5*.

### Autophagy Genes Control MHV68 Reactivation from Macrophages but Not from B Cells

Since MHV68 establishes latency in splenic B cells, we evaluated MHV68 reactivation in mice lacking *Atg5* in CD19-expressing cells (*Atg5<sup>ff</sup>-CD19cre*) (Conway et al., 2013; Miller et al., 2008; Pengo et al., 2013) at 16 days after infection, a time when reactivation from explanted B cells is robust enough to be quantified. MHV68 reactivated less efficiently from peritoneal macrophages from *Atg5<sup>ff</sup>-LysMcre* mice at this time point, but reactivation from splenic B cells was identical in *Atg5<sup>ff</sup>* and *Atg5<sup>ff</sup>-CD19cre* mice (Figure 2). Thus, *Atg5* deficiency does not control MHV68 reactivation in B cells.

### *Atg5* Is Not Required for Viral Replication or Establishment of Latent Infection

Defective reactivation could be due to a failure of MHV68 to replicate in *Atg5*-deficient macrophages or to establish or maintain latent infection. However, viral replication was normal in bone

marrow-derived macrophages (BMDMs) (Figure 3A) or PECs (Figure 3B) from *Atg5<sup>ff</sup>-LysMcre* mice, and there was no *Atg5*-associated defect in replication in vivo as measured by plaque assay (Figure 3C) or light emission in mice infected with luciferase-expressing MHV68 (M3FL) (Hwang et al., 2008; Reese et al., 2014) (Figure 3D). Further, the frequency of virus-positive PECs during chronic infection was not affected by *Atg5* mutation as measured by limiting dilution nested PCR targeting MHV68 viral *orf72* (Figure 3E) (Weck et al., 1999a, 1999b).

Thus decreased reactivation was not due to a role for *Atg5* in viral replication or latency establishment.

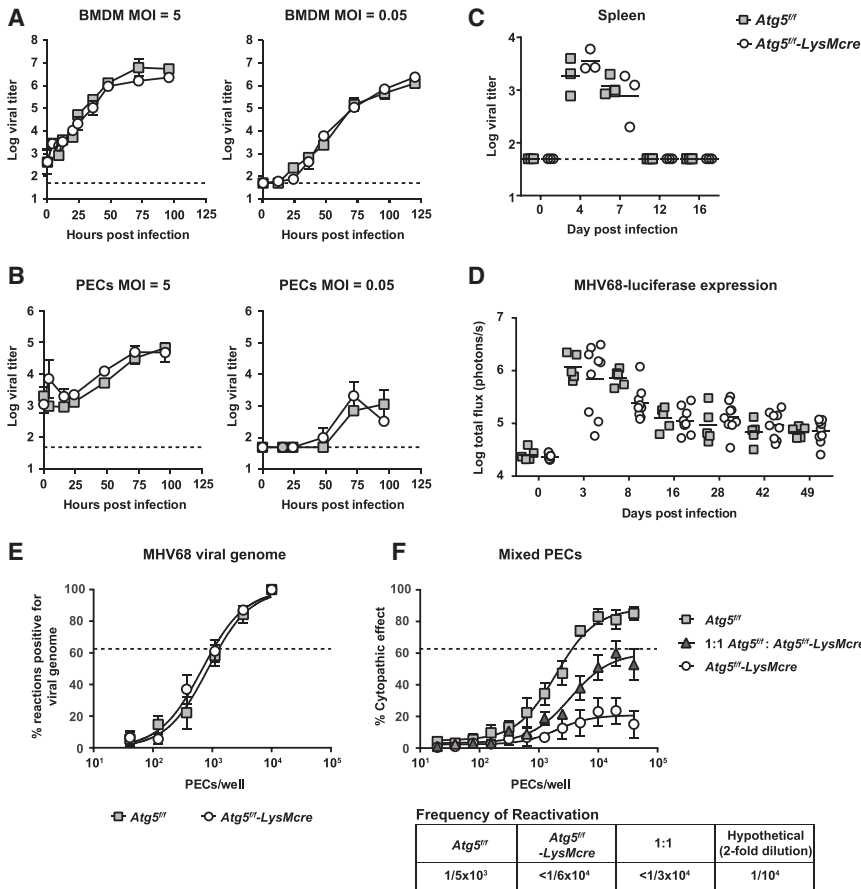
We next determined whether there was a paracrine factor from *Atg5<sup>ff</sup>-LysMcre* PECs that inhibited reactivation by comparing virus reactivation in PECs from *Atg5<sup>ff</sup>* and *Atg5<sup>ff</sup>-LysMcre* mice to reactivation from a 1:1 mixture of these cell populations (Figure 3F). In the 1:1 mixture, we observed greater than the 2-fold decrease expected if changes in reactivation were merely due to the dilution of *Atg5<sup>ff</sup>* cells by reactivation-defective *Atg5<sup>ff</sup>-LysMcre* PECs. This finding could be explained by the secretion of a paracrine factor, likely of immune origin, capable of blocking viral reactivation by reactivation-defective cell populations.

### *Atg5* Deficiency Increases Virus-Induced IFN- $\gamma$ -Dependent Macrophage Activation

We hypothesized that the immune response to MHV68 in *Atg5<sup>ff</sup>-LysMcre* mice might differ from that in wild-type mice. The frequency of latently infected macrophages was ca. 1/1,000 PECs (Figure 3E), indicating that changes in overall cell populations would be due to systemic effects of *Atg* gene deficiency rather than effects intrinsic to these rare infected cells. As expected, MHV68 infection increased inflammatory monocytes regardless of genotype (Figure S2A) (Barton et al., 2007). However, neutrophils were increased selectively in latently infected *Atg5<sup>ff</sup>-LysMcre* mice, consistent with a recent report of increased proliferation of peripheral neutrophils in *Atg5<sup>ff</sup>-LysMcre* mice (Rožman et al., 2015).

We next assessed the steady-state level of transcripts in peritoneal macrophages during chronic MHV68 infection using RNA-seq. Gene set enrichment analysis demonstrated changes in transcription specific to macrophages from MHV68-infected





**Figure 3. *Atg5* Is Not Required for Viral Replication or Establishment of Latent Infection**

(A and B) *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* BMDMs (A) or PECs (B) were infected with MHV68 at an MOI of 5 PFU/cell or 0.05 PFU/cell for 1 hr. Viral titers at the indicated time points were measured by plaque assay on an NIH 3T12 fibroblast monolayer. Data are the mean  $\pm$  SEM, and a paired t test over all time points yielded no significant differences. (C) *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice were infected with  $1 \times 10^6$  PFU MHV68 per mouse i.p., and splenic viral titers were measured by plaque assay at times indicated. Three to five mice per genotype were used for each time point. The limit of detection by plaque assay is 50 PFU and is represented by the dotted horizontal line (A–C). Data were analyzed by a paired t test at each time point, and no significant differences were observed. (D) *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice were infected with MHV68-M3FL, injected with D-luciferin, and imaged at the indicated times. Data were analyzed by a paired t test at each time point, and no significant differences were observed. (E) PECs were 3-fold serially diluted for a limiting dilution nested PCR assay specific for MHV68 ORF72 to measure the frequencies of MHV68 genome-containing PECs. For the limiting dilution PCR, n = 4 and data are the mean  $\pm$  SEM. Data are analyzed by a paired t test over all dilutions, and no significant differences observed. (F) *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice were infected with  $1 \times 10^6$  PFU MHV68 i.p. for 28 days, and LDA analysis was performed on PECs to measure virus reactivation. Data are combined from three biological replicate experiments with 3–5 mice pooled per experiment. PECs from *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice were mixed at a ratio of 1:1 and plated at the indicated ratios. Data are the mean  $\pm$  SEM.

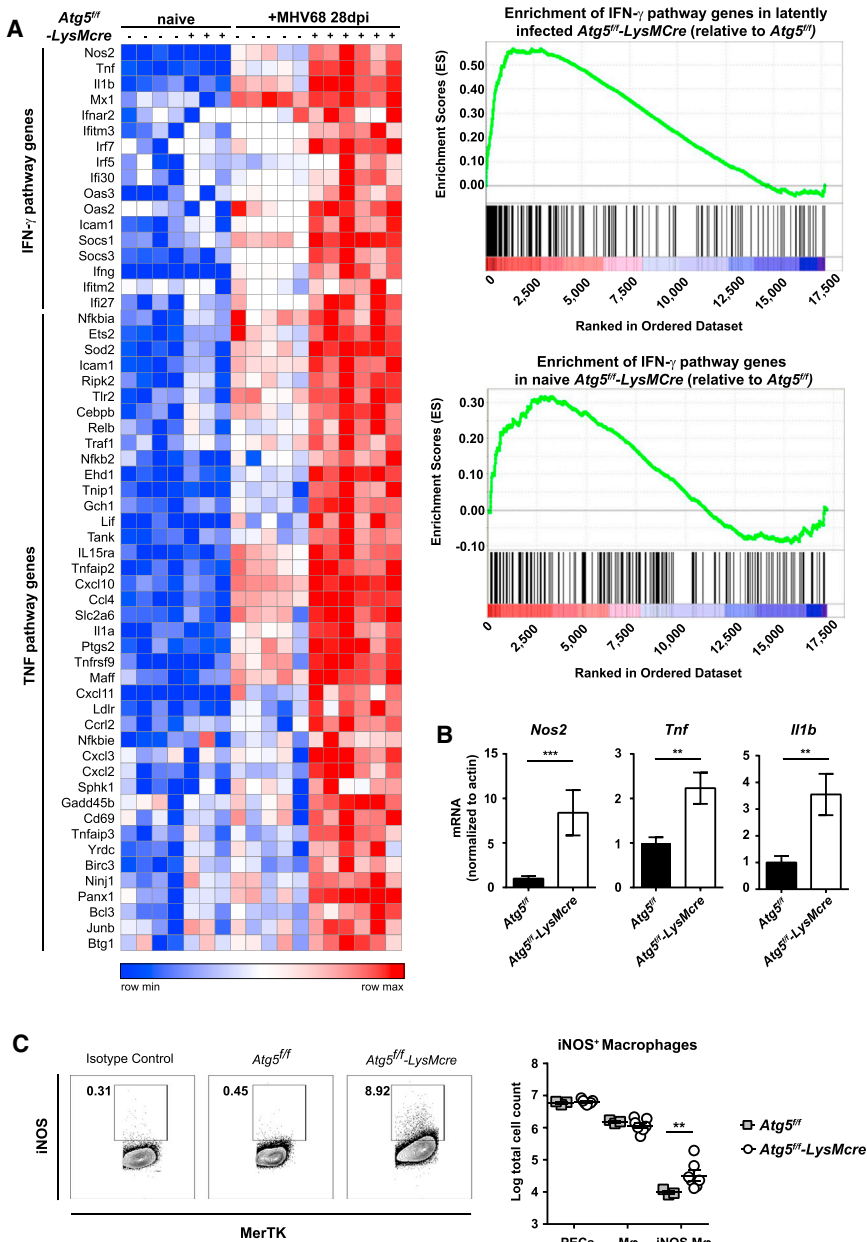
*Atg5<sup>fl/fl</sup>-LysMcre* mice, including upregulation of TNF and IFN- $\gamma$  response pathway genes (Figure 4A). A smaller increase in the expression of IFN- $\gamma$  response genes was observed in macrophages from uninfected *Atg5<sup>fl/fl</sup>-LysMcre* compared to *Atg5<sup>fl/fl</sup>* mice. qRT-PCR analysis validated RNA-seq results for *Nos2*, *Tnf*, and *I11b* (Figure 4B) and the interferon-regulated genes *Mx1* and *Oas2* (Figure S2B). Upregulation of IFN- $\gamma$  and TNF signaling pathways was also observed in macrophages from latently infected *Atg1611<sup>fl/fl</sup>-LysMcre* mice (Figure S2C). Peritoneal macrophages from infected *Atg5<sup>fl/fl</sup>-LysMcre* mice expressed increased levels of iNOS protein (Figures 4C and S2D). In addition, macrophages from uninfected *Atg5<sup>fl/fl</sup>-LysMcre* mice were hyper-responsive to IFN- $\gamma$  stimulation in vitro, expressing increased iNOS protein compared to cells from *Atg5<sup>fl/fl</sup>* mice (Figure S2E). Thus *Atg5<sup>fl/fl</sup>-LysMcre* macrophages have a small basal upregulation of IFN- $\gamma$  pathway genes and increased sensitivity to IFN- $\gamma$  stimulation. *Atg5*-dependent changes in macrophage gene expression in vivo were exacerbated by latent MHV68 infection even though only a very small proportion of these cells were latently infected.

**Increased T Cell-Derived IFN- $\gamma$  Inhibits MHV68 Reactivation in *Atg5<sup>fl/fl</sup>-LysMcre* Mice**

The gene expression data suggested a role for IFN- $\gamma$  in systemic inflammation, a hypothesis supported by the observation that

MHV68-infected *Atg5<sup>fl/fl</sup>-LysMcre* mice exhibited increased serum levels of IFN- $\gamma$  (Figure 5A). Explanted PECs from these mice secreted increased amounts of IFN- $\gamma$  (Figure 5B), indicating that a peritoneal cell type was producing increased IFN- $\gamma$ . To determine the source of IFN- $\gamma$ , we analyzed IFN- $\gamma$ -producing cell types including NK cells and CD4 and CD8 T cells. Total numbers of these cells were identical between *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice (Figure 6A). Depletion of NK cells throughout infection in *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice did not change virus reactivation (Figure S3A). We observed an increase in IFN- $\gamma$ -positive peritoneal CD4 T cells in latently infected *Atg5<sup>fl/fl</sup>-LysMcre* mice (Figure 6B). Depletion of CD4 and CD8 T cells decreased IFN- $\gamma$  secretion by explanted PECs, suggesting that T cells were a major source of IFN- $\gamma$  during chronic MHV68 infection of *Atg5<sup>fl/fl</sup>-LysMcre* mice (Figure 6C).

We did not find increased secretion of IL-12p70 or IL-18 or changes in cell surface expression of CD80, CD86, or MHC class II in macrophages from *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice that might explain these observations (Figures S3B and S3C). We adoptively transferred transgenic CD4 T cells expressing a T cell receptor (TCR) specific for MHV68 glycoprotein gp150 (Freeman et al., 2011) and CD8 T cells expressing a TCR specific for ovalbumin (Loh et al., 2012) into *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice followed by infection with MHV68 expressing ovalbumin



### Figure 4. *Atg5* Deficiency Increases Virus-Induced IFN- $\gamma$ -Dependent Macrophage Activation

(A) Gene set enrichment analysis was performed on RNA-seq data from naive and latent adherent peritoneal macrophages from *Atg5<sup>ff</sup>* and *Atg5<sup>ff</sup>-LysMcre* mice.

(B) Transcript levels of the indicated genes were measured in latently infected peritoneal macrophages from *Atg5<sup>ff</sup>* and *Atg5<sup>ff</sup>-LysMcre* mice. Data are analyzed by the non-parametric Mann-Whitney U test. \*\* $p < 0.01$ , \*\*\* $p < 0.005$ .

(C) Latently infected peritoneal macrophages were stained for intracellular iNOS and analyzed by flow cytometry. Each point represents a single mouse, and data are analyzed by the non-parametric Mann-Whitney U test. \*\* $p < 0.01$ .

betts et al., 2002; Weck et al., 1997) or establishment of latency (Sarawar et al., 1997; Steed et al., 2006; Tibbetts et al., 2002). IFN- $\gamma$  neutralization at the time of explant partially restored reactivation in PECs from *Atg5<sup>ff</sup>-LysMcre* mice (Figure S4A), but as the reactivation curve did not cross the Poisson distribution line, it was not possible to calculate the statistical significance for this reproducible effect (Figure S4B). There was no effect of anti-IFN- $\gamma$  treatment upon explant on reactivation from PECs from *Atg5<sup>ff</sup>* mice. When IFN- $\gamma$  was neutralized throughout the course of MHV68 infection in *Atg5<sup>ff</sup>-LysMcre* in vivo and in explant cultures, we observed increased reactivation in macrophages from *Atg5<sup>ff</sup>-LysMcre* mice to the level observed in macrophages from untreated *Atg5<sup>ff</sup>* mice (Figure 7), with no preformed virus detected in anti-IFN- $\gamma$  treated groups (Figure S4C). This procedure also increased reactivation of macrophages from *Atg5<sup>ff</sup>* mice. Differences in virus reactivation remained between *Atg5<sup>ff</sup>* and *Atg5<sup>ff</sup>-LysMcre* mice after neutralization of IFN- $\gamma$ , suggesting that this cytokine may not be the sole contributor to decreased reactivation observed in *Atg* gene-deficient mice. Thus IFN- $\gamma$  expression in vivo plays a role in limiting reactivation of MHV68 from cells derived from an autophagy-deficient environment.

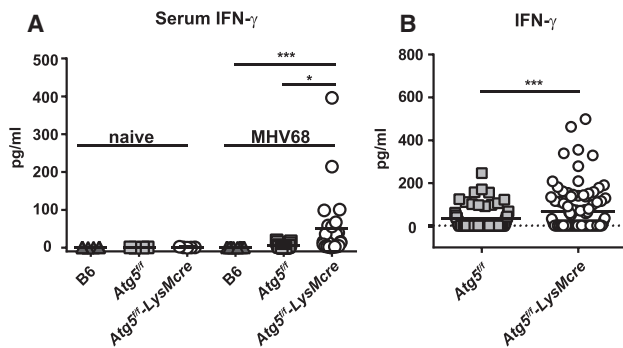
(Braaten et al., 2005). PECs were harvested 28 days later and stimulated with specific (gp150 or ovalbumin) and control (influenza NP<sub>366-374</sub>) peptides (Figures S3D and S3E). Virus-specific CD4 and CD8 peritoneal T cells expressed IFN- $\gamma$  after antigen stimulation similarly regardless of whether they came from *Atg5<sup>ff</sup>* or *Atg5<sup>ff</sup>-LysMcre* mice, indicating that enhanced IFN- $\gamma$  production is due to an in vivo effect not duplicated in ex vivo T cell stimulation assays.

### IFN- $\gamma$ Neutralization Rescues MHV68 Reactivation in PECs from *Atg5<sup>ff</sup>-LysMcre* Mice

Data on elevated IFN- $\gamma$  levels were of interest because this cytokine inhibits MHV68 reactivation from latency (Goodwin et al., 2010; Steed et al., 2006, 2007) with minimal effects on acute replication of MHV68 (Dutia et al., 1997; Sarawar et al., 1997; Tib-

### DISCUSSION

We report that deletion of *Atg* genes in LysM-expressing cells resulted in decreased efficiency of MHV68 reactivation from latent infection and increased virus-triggered systemic inflammation, including high circulating IFN- $\gamma$  levels and expression of TNF and IFN- $\gamma$  pathway genes in macrophages. IFN- $\gamma$  is likely an important link between systemic inflammation and decreased reactivation from latency, as this cytokine potently inhibits



**Figure 5. Elevated IFN- $\gamma$  in *Atg5<sup>fl/fl</sup>-LysMcre* Mice Drives Macrophage Activation**

(A) Serum levels of IFN- $\gamma$  were measured by Luminex xMAP technology 42 days after infection. Each data point represents one mouse. Data were analyzed by a one-way ANOVA with Kruskal-Wallis test with multiple comparisons. \* $p < 0.05$ , \*\*\* $p < 0.005$ .

(B) 40,000 PECs pooled from three *Atg5<sup>fl/fl</sup>* mice or *Atg5<sup>fl/fl</sup>-LysMcre* mice were plated onto a monolayer of MEFs for 24 hr in a 96-well plate. IFN- $\gamma$  levels in the supernatants were measured by a sandwich ELISA. The data are four biological replicate experiments with 24 technical replicate wells per sample. Each data point represents a technical replicate and was analyzed by the non-parametric Mann-Whitney U test. \*\*\* $p < 0.005$ .

reactivation from latency in macrophages *in vivo* and *in vitro* (Goodwin et al., 2010; Steed et al., 2006, 2007), and neutralization of IFN- $\gamma$  increased viral reactivation in macrophages from mice lacking *Atg5* in LysM-expressing cells. Thus, *Atg* genes in myeloid cells can influence the course of chronic viral infection through effects on the overall inflammatory state of the host and are responsible for limiting systemic inflammation during chronic herpesvirus infection.

### Role of *Atg* Genes in Myeloid Cells in Control of Herpesvirus Reactivation

Deleting *Atg* genes including *Atg5*, *Atg16l1*, *Atg7*, *Atg3*, *Atg14*, *beclin 1*, and *Fip200* in myeloid cells resulted in inhibition of MHV68 reactivation from latently infected macrophages. However, mice lacking *Atg4b* had normal frequencies of reactivation. This discrepancy may be attributed to the existence of multiple isoforms of *Atg4* (Choi et al., 2014; Mariño et al., 2010). The involvement of all of these *Atg* genes in control of reactivation is most consistent with a requirement for canonical autophagy, a process requiring all of the *Atg* genes tested (Levine et al., 2011; Martinez et al., 2015) in control of chronic virus infection. The involvement of *Atg14* and *Fip200* is informative, as these genes are not required for LAP (Martinez et al., 2015). This differs from other situations in which canonical autophagy-independent functions of *Atg* genes control pathogen infection (Choi et al., 2014; Hwang et al., 2012; Reggiori et al., 2010; Zhao et al., 2008).

### Cell-Intrinsic Versus Cell-Extrinsic Effects of *Atg* Genes on Viral Infection

IFN- $\gamma$  inhibits murine norovirus growth in macrophages in a manner dependent on *Atg5*, *Atg7*, and *Atg16l1*, but not on *Atg14* or the degradative function of canonical autophagy (Hwang et al., 2012). *Atg* genes can also have pro-viral actions

in infected cells, such as in the release of picornaviruses from infected cells (Alirezai et al., 2012; Taylor and Kirkegaard, 2008) and the replication of Dengue virus, coronavirus, and hepatitis C virus (Dreux et al., 2009; Heaton et al., 2010; Reggiori et al., 2010). In these situations *Atg* genes act directly in the infected cell. In contrast, we found no evidence for a cell-intrinsic role for *Atg5* in MHV68 replication. Instead, *Atg* gene deficiency enhanced virus-triggered systemic immune activation during chronic infection, which resulted in decreased reactivation from latency. These data reveal inhibition of systemic inflammation as an additional mechanism through which *Atg* genes regulate viral infection.

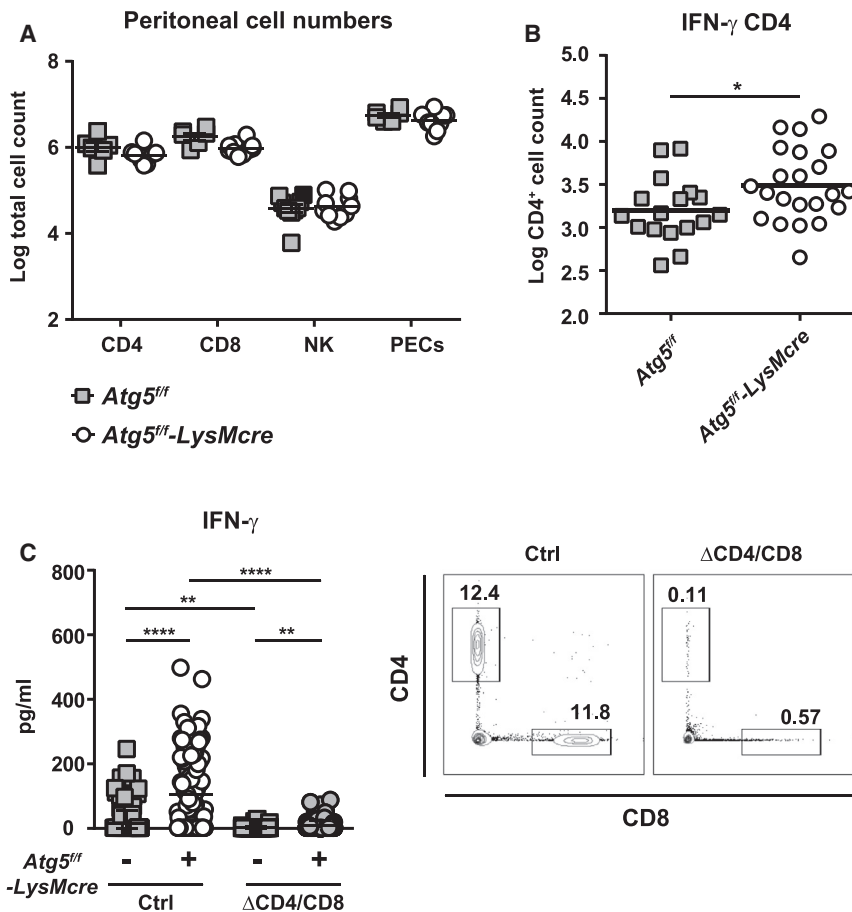
MHV68 encodes a viral Bcl-2 (v-Bcl-2) protein reported to inhibit both autophagy and apoptosis. Analysis of MHV68 v-Bcl-2 mutants in BALB/c mice supports a role for v-Bcl-2-mediated inhibition of autophagy in maintenance of latency, while the anti-apoptotic function fosters viral reactivation (E et al., 2009). However, in C57BL/6J mice, full deletion of v-Bcl-2 only modestly inhibits viral reactivation from latency without changing the frequency of latently infected cells (Gangappa et al., 2002; Loh et al., 2005). Using C57BL/6J mice, we observed profound effects of deletion of *Atg* genes in the myeloid lineage on reactivation but not establishment or maintenance of viral latency. Since deletion of v-Bcl-2 (which should increase autophagy in infected macrophages) and the deletion of *Atg* genes in myeloid cells (which inhibits autophagy in both infected and uninfected cells) both result in decreased reactivation, the data strongly support the concept that the dominant effects observed here are due to *Atg* gene effects outside of the infected cell. This further supports our conclusion that regulation of virus-induced systemic inflammation is an important role for *Atg* genes.

### Role of Autophagy and *Atg* Genes in Controlling Inflammation

Numerous studies have identified *Atg* genes and canonical autophagy in macrophages as key regulators of inflammation (Abdel Fattah et al., 2015; Deretic et al., 2015; Kanayama et al., 2015; Levine et al., 2011). While we measured events that occur in macrophages such as gene expression and viral reactivation, *LysMcre*-mediated gene deletion occurs in multiple cell types including neutrophils and some dendritic cells (Clausen et al., 1999; Jakubczik et al., 2008). Therefore, the effects on macrophage-dependent assays presented here could be due to effects of *Atg* genes in either macrophages or other LysM-expressing cell types that regulate inflammation triggered by chronic virus infection.

We found that peritoneal macrophages from uninfected *Atg5<sup>fl/fl</sup>-LysMcre* mice exhibited a significant upregulation of IFN- $\gamma$  response pathway genes, an effect likely due to increased MHV68 infection-driven IFN- $\gamma$  expression in CD4 T cells. Macrophages from uninfected *Atg5<sup>fl/fl</sup>-LysMcre* mice were hyper-responsive to IFN- $\gamma$  treatment. Interestingly, *Atg5*-deficient BMDMs have largely normal responses to IFN- $\gamma$  (Hwang et al., 2012; Zhao et al., 2008), strongly indicating that events *in vivo* are triggering *Atg* gene-controlled inflammation, even without the stimulus of chronic virus infection. These findings suggest that *Atg* genes are required to limit basal





**Figure 6. Increased T Cell-Derived IFN- $\gamma$  Inhibits MHV68 Reactivation in *Atg5<sup>fl/fl</sup>-LysMcre* Mice**

(A) Total numbers of peritoneal CD4, CD8, and NK cells were measured by flow cytometry in latently infected *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* mice. Each point represents a single mouse, and data are analyzed by the non-parametric Mann-Whitney U test. No significant differences were observed. (B) Absolute cell numbers and % of cells of IFN- $\gamma$ -positive CD4 were measured in latently infected *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre* PECs using intracellular staining after 5 hr of Brefeldin A and monensin A treatment ex vivo. Data are three independent biological replicate experiments and analyzed by non-parametric Mann-Whitney U test. \* $p = 0.02$ . (C) CD4 and CD8 T cells were depleted 2 days prior to harvesting PECs. Latently infected PECs were plated onto MEFs for 24 hr, and IFN- $\gamma$  supernatant levels were measured by ELISA. Efficiency of depletion was measured by flow cytometry. Data were analyzed by a one-way ANOVA with Kruskal-Wallis test with multiple comparisons. \*\* $p < 0.01$ , \*\*\* $p < 0.005$ , \*\*\*\* $p < 0.001$ .

inflammation and that a trigger such as chronic viral infection can amplify the abnormal basal inflammation observed when myeloid cells lack *Atg* genes. The fact that basal lung inflammation is also inhibited by *Atg* genes (Lu et al., 2016), and that the lung inflammation that results from deletion of these genes in myeloid cells prevents lethal influenza virus infection, underlines the general role of *Atg* genes in regulating infection through effects on inflammation.

### Implications for Disease and the Biology of Chronic Viral Infections

We and others have documented the consequences of chronic viral infections and the mammalian virome in shaping immune responses to secondary infections and other challenges (Barton et al., 2007; MacDuff et al., 2015; Osborne et al., 2014; Reese et al., 2014; Stelekati et al., 2014; Virgin, 2014; Virgin et al., 2009). For example, chronic MHV68 infection induces prolonged low-level production of IFN- $\gamma$  and activation of macrophages in normal mice, resulting in symbiotic resistance to bacterial infection (Barton et al., 2007). Data presented here indicate that this process is enhanced in mice lacking *Atg5* or *Atg16/1* in myeloid cells. Our data suggest that autophagy plays a key role in limiting inflammation caused by common chronic viral infections, thereby protecting the host from diseases worsened by chronic inflammation. This may be significant, as most or all humans carry multiple chronic virus infections, and systemic inflamma-

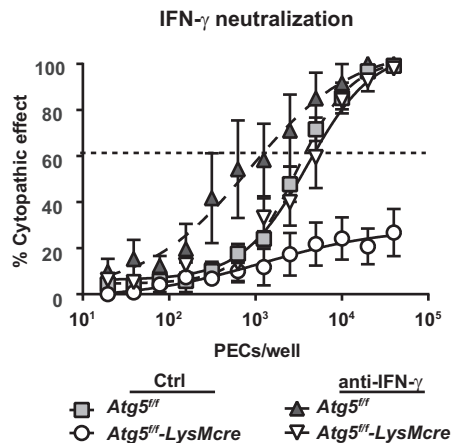
tion is linked to many human diseases including cancer, diabetes, cardiovascular disease, and auto-immunity (Virgin, 2014). It is important to note that all experiments presented here are loss-of-function in design and therefore do not demonstrate that induction of autophagy would limit basal or virus-induced inflammation. However, our data support testing the hypothesis that basal and virus-triggered inflammation may be lessened by induction of autophagy.

One could speculate that chronic herpesvirus infection sets the stage, in an *Atg* gene-dependent manner, for protective effects of innate or adaptive immunity and harmful effects of inflammation or auto-immunity in persons with specific genetic constitutions. For example, chronic MHV68 infection complemented genetic immunodeficiency due to mutation of *Hoi1-1*, but did so at the cost of increased systemic inflammation (Boisson et al., 2012; MacDuff et al., 2015). Studies presented here combined with prior findings support the “virus plus host gene” concept that significant physiologic consequences develop when chronic virus infection intersects with host genetic susceptibility (Cadwell et al., 2008, 2010; Kernbauer et al., 2014; Virgin, 2014).

### EXPERIMENTAL PROCEDURES

#### Mice

*Atg5<sup>fl/fl</sup>*, *Atg5<sup>fl/fl</sup>-LysMcre*, and *Atg5<sup>fl/fl</sup>-CD19cre* mice were generated as described previously in an enhanced barrier facility (Miller et al., 2008; Zhao et al., 2008). *Atg16/1<sup>fl/fl</sup>-LysMcre* (Hwang et al., 2012), *Atg7<sup>fl/fl</sup>-LysMcre* (DeSelm et al., 2011), *Atg14<sup>fl/fl</sup>-LysMcre* (Choi et al., 2014), *Atg3<sup>fl/fl</sup>-LysMcre* (Choi et al., 2014), *beclin 1<sup>fl/fl</sup>-LysMcre* (Sanjuan et al., 2007), and *Fip200<sup>fl/fl</sup>-LysMcre* (Gan et al., 2006) were generated in the same way as *Atg5<sup>fl/fl</sup>-LysMcre*. *p62<sup>-/-</sup>* (Komatsu et al., 2007) and *Atg4b<sup>-/-</sup>* (Mariño et al., 2010) mice were described elsewhere. All mice used for experimental procedures were 8–10 weeks of age



**Figure 7. IFN- $\gamma$  Neutralization Rescues MHV68 Reactivation in PECs from *Atg5<sup>fl/fl</sup>-LysMcre* Mice**

IFN- $\gamma$  was neutralized with 250  $\mu$ g/mouse i.p. every 7 days during the course of MHV68 infection for 28 days in *Atg5<sup>fl/fl</sup>* and *Atg5<sup>fl/fl</sup>-LysMcre*, and virus reactivation in PECs was measured ex vivo in the presence of anti-IFN- $\gamma$  antibody. Data were combined from three biological experiments with 3–5 mice pooled per experiment and represented with the mean  $\pm$  SEM.

and sex-matched littermates. Unless specified, all mice were infected with  $1 \times 10^6$  PFU MHV68 intraperitoneally (i.p.). All mice were housed and bred at Washington University in St. Louis in specific pathogen-free conditions in accordance with federal and university guidelines, and protocols were approved by the Animal Studies Committee of Washington University under protocol number 20140244.

#### LDA and LD-PCR

LDAs to measure the frequency of MHV68 ex vivo reactivation were performed as previously described (Tibbetts et al., 2002, 2003; Weck et al., 1996). Briefly, spleens and PECs were harvested and pooled from 3–5 latently infected mice per group. Serial dilutions of splenocytes or PECs were plated on a monolayer of MEFs, and cytopathic effect was used to assess the presence of virus after 3 weeks ex vivo. Applying the Poisson distribution, the horizontal line at 63.2% measures the frequency at which one reactivation event is likely to occur per well. To assess the presence of preformed infectious virus in cells, cells were mechanically disrupted with 0.5-mm silica beads on the mini Bead-beater-8 (Biospec). To determine the frequency of cells containing MHV68 genome, a single-copy sensitivity nested PCR in limiting dilutions (LD-PCR) for MHV68 gene *ORF72/v-cyclin* was performed using PECs from latently infected mice. Cells were serially diluted in a solution containing uninfected NIH 3T12 cells to maintain a total number of  $10^4$  cells per PCR reaction. Samples were digested overnight with proteinase K, and two rounds of PCR were performed using six dilutions per sample with 12 reactions per dilution. Single copies of a plasmid containing *ORF72* were used as positive controls. Products were visualized on a 1.5% agarose gel. Data points for LDA and LD-PCR represent the mean and the standard error of the mean for at least three replicate experiments, using 3–5 mice pooled per experiment. Using GraphPad Prism, a sigmoidal dose curve with a nonvariable slope was applied to data points. Frequencies of reactivation or viral genome-carrying cells were obtained by calculating the cell density at which 63.2% of the wells were positive based on the Poisson distribution. A paired t test was applied over all dilutions.

#### Cytokine Measurements by ELISA or Multiplex Cytokine Arrays

Sera from naive or infected mice were collected by cardiac puncture. Cytokines from sera were measured using a multiplex array (eBioscience) on a Bio-plex 200 (Bio-Rad). IFN- $\gamma$  (BioLegend, 430803), IL-12p70 (BD Biosciences, 555256), and IL-18 (eBiosciences, BMS618) from supernatants of latently infected PECs were measured by a sandwich ELISA after plating for 24 hr on MEF monolayers.

#### Depletion of IFN- $\gamma$ , NK, and T Cells In Vivo

For depletion of IFN- $\gamma$ , clone H22 was used at 250  $\mu$ g/mouse, injected i.p. every 7 days. Control hamster IgG (PIP) was used for control groups. PECs were isolated in the presence of 10  $\mu$ g/ml antibody and plated in the presence of these antibodies for evaluation of reactivation. NK cells were depleted with anti-NK1.1 (clone PK136) at 200  $\mu$ g/mouse injected i.p. every 5 days beginning at 2 days prior to infection and continued until sacrifice. Mouse anti-rabbit isotype control was used for control groups. Reactivation was measured in the presence of 10  $\mu$ g/ml antibody. To deplete T cells, CD4 (clone YTS 101.5) or CD8 (clone H35 17.2) depleting antibodies were injected 500  $\mu$ g/mouse i.p. every other day (Chachu et al., 2008), 2 days before sacrifice. Isotype-matched control (SFR-DR5, IgG2b) was administered to control groups. Additional experimental procedures are available in the Supplemental Information.

#### ACCESSION NUMBERS

RNA-seq data are available at the European Nucleotide Archive: PRJEB10074.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2015.12.010>.

#### AUTHOR CONTRIBUTIONS

H.W.V., S.P., D.R.G., B.L., and T.A.R. conceived experiments. S.P. and H.W.V. wrote the manuscript. All authors reviewed the manuscript. S.P. performed all experiments. M.N.A., M.D.B., C.D., E.L., and X.Z. performed specific experiments. M.L.F., T.S., S.A., J.-L.G., J.M., Y.-W.H., and M.A.B. provided reagents. B.L., D.R.G., S.A.H., M.N.A., and H.W.V. provided expertise and feedback.

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