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Homeostatic Control of Innate Lung Inflammation by Vici Syndrome Gene *Epg5* and Additional Autophagy Genes Promotes Influenza Pathogenesis

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SUMMARY

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AUTHOR CONTRIBUTIONS

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Mutations in the autophagy gene *EPG5* are linked to the multisystem human disease Vici syndrome, which is characterized in part by pulmonary abnormalities, including recurrent infections. We found that *Epg5*-deficient mice exhibited elevated baseline innate immune cellular and cytokine-based lung inflammation and were resistant to lethal influenza virus infection. Lung transcriptomics, bone marrow transplantation experiments, and analysis of cellular cytokine expression indicated that *Epg5* plays a role in lung physiology through its function in macrophages. Deletion of other autophagy genes including *Atg14, FIP200, Atg5*, and *Atg7* in myeloid cells also led to elevated basal lung inflammation and influenza resistance. This suggests that *Epg5* and other *Atg* genes function in macrophages to limit innate immune inflammation in the lung. Disruption of this normal homeostatic dampening of lung inflammation results in increased resistance to influenza, suggesting that normal homeostatic mechanisms that limit basal tissue inflammation support some infectious diseases.

INTRODUCTION

epg-5 was identified as an autophagy (*Atg*) gene in genetic screens for loss-of-function defects in autophagy substrate degradation during *C. elegans* embryogenesis (Tian et al., 2010). Recessive mutations in human *EPG5* are causally associated with the multisystem disorder Vici syndrome (Cullup et al., 2013). Some features of Vici syndrome, including abnormalities in autophagy, neurodegeneration and myopathy are recapitulated in mice deficient for *Epg5* (Zhao et al., 2013b). Vici syndrome patients exhibit variable immune system abnormalities and recurrent bronchopulmonary infections (Ehmke et al., 2014; Finocchi et al., 2012), but the role of *EPG5* in immunity and in the lung has not been defined in detail.

Macroautophagy (canonical autophagy herein) is a process by which cells degrade cytoplasmic cargo captured within double membrane-bound autophagosomes (Green and Levine, 2014; Levine et al., 2011). Canonical autophagy is triggered through a pre-initiation complex composed of a core of ULK1/2, ATG13, and FIP200 proteins. The pre-initiation complex activates the initiation complex consisting of a core of proteins including ATG14, Beclin 1, VPS34, and VPS15 whose concerted action triggers generation of the isolation membrane. Generation of the mature double membrane-bound autophagosome containing captured cargo from the isolation membrane involves two ubiquitin-like protein conjugation systems which utilize ATG7 as the common E1 enzyme. The first system, involving proteins ATG10, ATG4 and ATG3, conjugates LC3 family members to phosphatidyl-ethanolamine creating LC3-II from LC3-I. The second system conjugates ATG12 to ATG5 which then complexes with ATG16L1 to form an E3-like complex directing LC3-II to the autophagosome. Binding of adapter molecules such as p62 specifically target substrates to the interior of the autophagosome. Fusion of autophagosomes and lysosomes results in degradation of captured cytoplasmic constituents. Mammalian *Epg5* is essential for basal autophagy and functions in the formation of degradative autolysosomes (Zhao et al., 2013a).

Atg genes and proteins have been linked to inflammation during infection (Deretic, 2012; Levine et al., 2011; Saitoh et al., 2008), and regulation of the adaptive immune system through effects in both B and T cells (Chen et al., 2014; Conway et al., 2013; Miller et al.,

2008; Pei et al., 2015; Pengo et al., 2013; Pua et al., 2009; Puleston et al., 2014; Stephenson et al., 2009; Xu et al., 2014). *Atg* genes also play important roles in macrophages and regulate inflammasome activity resulting in increased secretion of IL-1β and IL-18 upon lipopolysaccharide stimulation (Dupont et al., 2011; Nakahira et al., 2011; Saitoh et al., 2008; Shi et al., 2012). Some, but not all, *Atg* genes function in a Toll-like receptor or immunoglobulin receptor-triggered pathway called LC3-associated phagocytosis (LAP) (Henault et al., 2012; Huang et al., 2009; Martinez et al., 2011; Martinez et al., 2015; Sanjuan et al., 2007). The *Atg* genes *Atg5* and *Atg7* in Lysozyme-M-cre recombinase (LysMcre) expressing cells have recently been reported to protect against spontaneous lung inflammation (Abdel et al., 2015; Kanayama et al., 2015). Furthermore, homozygous deletion of *Atg5* is associated with retinal and lung inflammation in developing embryos (Qu et al., 2007). Consequences of the regulation of tissue inflammation by *Atg* genes for infection have not been evaluated, and the role of canonical autophagy versus other *Atg* gene-dependent processes such as LAP in the lung has not been identified.

Influenza A viruses (IAV) are negative-sense viruses that infect humans and animals. Lung inflammation during IAV infection is a double-edged sword; optimal cytokine levels exert protective effects against viral replication and disease, while excessive cytokine and cellular inflammation results in IAV-induced lung damage (Iwasaki and Pillai, 2014; McNab et al., 2015; Ramos and Fernandez-Sesma, 2015; Teijaro et al., 2014). The timing of cytokine expression and cellular inflammation versus viral replication is a critical determinant of the outcome of infection since pre-existing inflammation can enhance resistance to IAV (Ishikawa et al., 2012; Samarasinghe et al., 2014). The relationship between autophagy and influenza is incompletely understood. Autophagy can be induced by IAV infection, and is reportedly involved in viral replication (Lupfer et al., 2013; Zhou et al., 2009). *Atg* genes have been implicated in viral entry, viral release, and cell death during IAV infection (Beale et al., 2014; Pirooz et al., 2014; Sun et al., 2012). Furthermore, IAV can inhibit degradation by autophagosomes (Gannage et al., 2009). How host autophagy affects IAV pathogenesis *in vivo* is not understood.

In this study, we characterized the role of *Epg5* in lung inflammation and during IAV infection, discovering that *Epg5*$^{-/-}$ mice are highly resistant to influenza. Prior to infection, *Epg5*$^{-/-}$ mice exhibited profound cellular and cytokine-based lung inflammation, including elevated expression of cytokines associated with influenza resistance. Bone marrow transplantation studies, genetic studies, transcriptional profiling, and cytokine expression analysis suggested that *Epg5* controls innate lung inflammation through effects in macrophages. Consistent with this hypothesis, deletion of additional *Atg* genes including *Atg14*, *FIP200*, *Atg5*, and *Atg7* in myeloid cells resulted in increased lung inflammation and influenza resistance. Together, these studies demonstrate *Epg5*, and additional *Atg* genes play a critical role in controlling lung inflammation through actions in myeloid cells especially macrophages. Loss of this function results in disruption of lung homeostasis, increased inflammation, and sets the stage for resistance to influenza. Susceptibility to IAV infection is therefore due in part to the normal anti-inflammatory effects of *Atg* genes in the lung. Interestingly, in a companion paper we found that certain *Atg* genes act in myeloid cells to prevent systemic inflammation during chronic herpesvirus infection (Park, *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.

RESULTS

Epg5 Deficiency Protects Mice from Influenza

While the majority of Vici syndrome patients have recurrent pulmonary infections (Ehmke et al., 2014; Finocchi et al., 2012), the role of *EPG5* in inflammation and infection in the lung has not been defined in detail. We therefore evaluated $Epg5^{-/-}$ mice for abnormal responses to respiratory virus infection. Mice were infected with California H1N1 IAV and monitored for morbidity and mortality (Figure 1A). $Epg5^{+/+}$ and $Epg5^{+/-}$ mice were susceptible to influenza, while $Epg5^{-/-}$ mice were completely resistant. Influenza resistance was also observed in response to the mouse-adapted H1N1 IAV strain PR8 (Figure 1B). Autophagy deficiency results in the accumulation of p62/SQSTM1 (p62), which is associated with altered tissue function and NFκB activation (Duran et al., 2004; Komatsu et al., 2007). However, deletion of p62 on the $Epg5^{-/-}$ mouse background did not reverse the influenza resistance phenotype (Figure S1A). $Epg5^{-/-}$ mouse resistance to IAV infection correlated with decreased viral titers 48 hours post-infection (hpi, Figure 1C). Immunohistochemistry (IHC) staining for the structural nucleocapsid protein (flu NP) in epithelial and parenchymal cells was also reduced in $Epg5^{-/-}$ mice 48 hpi (Figure 1D). Since the autophagy protein UVRAG influences early stages of the viral lifecycle (Pirooz et al., 2014), we evaluated virus entry and gene expression in $Epg5^{-/-}$ murine embryonic fibroblast (MEF) cells and bone marrow derived macrophages (BMDMs). The number of cells expressing flu NP was not altered in either cell type by *Epg5* deficiency (Figure S1B).

Elevated Cellular Lung Inflammation in $Epg5^{-/-}$ Mice

Given that $Epg5^{-/-}$ mice control viral titers early post infection, we evaluated uninfected $Epg5^{-/-}$ mice for lung abnormalities. The lungs of uninfected $Epg5^{-/-}$ mice exhibited striking histologic abnormalities including the appearance of lymphoid aggregates, large macrophage-like cells, polymorphonuclear leukocytes (PMNs) (Figure 2A), and mucus cell metaplasia (Figure S2A). Analysis of cells from bronchoalveolar lavage fluids (BAL) revealed abnormal macrophages and abundant PMNs (Figure 2A). IHC staining identified $B220^{+}$ B cells as the major cell type in the lymphoid aggregates and confirmed the presence of increased $F4/80^{+}$ macrophages (Figure S2B). Increased cellular inflammation and abnormal macrophage appearance was unlikely due to infection by known mouse pathogens, as serologic responses to these agents were not detected in adult $Epg5^{+/+}$ or $Epg5^{-/-}$ animals despite the presence of serum IgG (Table S1), and the fact that these animals were capable of generating anti-IAV antibodies after infection (data not shown). The histology of other organs, including liver, kidney, spleen, ileum, and colon, appeared normal (data not shown).

These histological observations were confirmed by flow cytometric analysis. Lungs of $Epg5^{-/-}$ mice contained more total cells and increased percentages of alveolar macrophages (AMs), PMNs, and eosinophils but not T or B cells (Figure 2B). AMs in $Epg5^{-/-}$ mouse lungs exhibited atypical surface marker expression. While $Epg5^{+/+}$ AMs were

CD11c^{+}SiglecF^{+}CD64^{+}CD11b^{low}, representing mature AMs, these cells comprised only 3% of AMs in *Epg5*$^{-/-}$ mouse lungs (Figure 2C). Instead, up regulation of CD11b was seen in AM-like cells in *Epg5*$^{-/-}$ mouse lungs, a population not observed in *Epg5*$^{+/+}$ mice (Figure 2C). These abnormalities in cell populations and surface marker expression were observable as early as nine days after birth (Figure S2C). However, at one day after birth, numbers of total cells, pre-AMs and PMNs were equivalent between *Epg5*$^{-/-}$ and *Epg5*$^{+/+}$ mice (Figure S2D).

Alterations in Lung Homeostasis in *Epg5*$^{-/-}$ Mice are Driven by Hematopoietic Cells

We generated bone marrow chimeras to determine whether expression of *Epg5* in radiation-sensitive or -resistant cells was responsible for the inflammatory lung phenotype. We transferred CD45.1 wild type (WT) bone marrow (BM) cells into lethally-irradiated CD45.2 *Epg5*$^{+/+}$ and *Epg5*$^{-/-}$ recipients, and CD45.2 *Epg5*$^{-/-}$ BM cells into lethally irradiated CD45.1 WT recipients. The hematopoietic compartment in recipient mice was efficiently reconstituted by BM transplantation, as demonstrated by the ratio of CD45.1 to CD45.2 blood cells in recipient mice (Figure S2E). AMs developed normally in *Epg5*$^{-/-}$ mice receiving CD45.1 *Epg5*$^{+/+}$ BM (Figure S2F). Conversely, CD45.1 *Epg5*$^{+/+}$ receiving *Epg5*$^{-/-}$ BM exhibited the distinct AM-like CD11b^{+} cell population previously seen in *Epg5*$^{-/-}$ mice (Figure S2F). These data indicate that *Epg5* deficiency in the hematopoietic compartment drives abnormal macrophage differentiation in the lungs of *Epg5*$^{-/-}$ mice.

Epg5 Deficiency Enhances the Innate Immune Response to Influenza

BM transplantation reconstitutes both myeloid and lymphoid lineages. To test if influenza resistance associated with *Epg5* deficiency requires adaptive immune cells, we generated *Rag1*$^{-/-}$*Epg5*$^{-/-}$ mice, which lack mature B and T cells. We observed increased lung cellular inflammation and abnormal macrophage differentiation in *Rag1*$^{-/-}$ *Epg5*$^{-/-}$ mice by histology, analysis of BAL, and flow cytometry, though lymphoid aggregates were absent as expected (Figure 3A-C). We challenged *Rag1*$^{-/-}$*Epg5*$^{-/-}$ mice with IAV and found that, while adaptive immunity is essential for long-term survival after IAV infection, deletion of *Epg5* in the absence of adaptive immunity reduced morbidity and prolonged survival (Figure 3D). These data indicate that *Epg5* deficiency protects mice from influenza and causes lung inflammation through altered innate immunity.

Lung Transcriptome Analysis Identifies Macrophages as Central to Inflammation in *Epg5*$^{-/-}$ Mice

We used RNA-Seq to identify dysregulated pathways in in both *Epg5*$^{-/-}$ and *Rag1*$^{-/-}$*Epg5*$^{-/-}$ mice. Analysis of steady-state RNA levels in the lungs of *Epg5*$^{-/-}$ mice revealed up regulation of transcripts associated with the interferon-γ (IFNγ) pathway and both M1 and M2 macrophage differentiation (Figure S3A and S3C). These same pathways were up regulated in *Rag1*$^{-/-}$*Epg5*$^{-/-}$ mice (Figure S3B and S3D), again indicating that the effects of *Epg5* deficiency were due to changes in innate immunity. These lung transcriptome changes, together with altered cell populations, abnormal expression of AM cell surface markers, and BM transplantation results, further indicate that macrophages are

abnormal in *Epg5*$^{-/-}$ mice, and suggested that the macrophage may be a central player in the lung inflammation and influenza resistance in *Epg5*$^{-/-}$ mice.

Epg5 Regulates Basal Expression of Multiple Cytokines in the Lung

Susceptibility and resistance of mice to influenza has been linked to a number of cytokines and chemokines (Iwasaki and Pillai, 2014; McNab et al., 2015; Ramos and Fernandez-Sesma, 2015). Cytokines such as GM-CSF (Huang et al., 2011; Schneider et al., 2014; Sever-Chroneos et al., 2011), IL-6 (Dienz et al., 2012), types I, II, and III interferons (McNab et al., 2015), and IL-1β (Ichinohe et al., 2009; Schmitz et al., 2005; Thomas et al., 2009) are involved in protection against influenza. In contrast, TNFα likely contributes to the pathogenesis of IAV-induced disease (Shi et al., 2013; Szretter et al., 2007). We hypothesized that cytokines important for the control of influenza are elevated in lungs of uninfected *Epg5*$^{-/-}$ mice. qPCR analysis revealed elevations in a range of cytokine transcripts including GM-CSF, TNFα, IL-1β, IL-6, MCP-1, and IL-13 (Figure 4A). These changes were selective, as levels of other cytokines including IL-4, IFNα, IFNβ, and IFNγ were not elevated (Figure 4A and data not shown). Elevated cytokine transcript expression was unique to the lung, as levels of TNFα and IL-1β were unchanged in liver, ileum, colon, spleen, and kidney (Figure 4B). Increased cytokines were also observed in *Rag1*$^{-/-}$*Epg5*$^{-/-}$ mouse lungs (Figure 4C), indicating that these changes were innate immune in nature. These data indicate that *Epg5* controls the production of multiple pulmonary cytokines including several associated with influenza resistance. The presence of these cytokines prior to infection may reasonably contribute to the influenza resistance observed in *Epg5*$^{-/-}$ mice.

Epg5 Deficiency Causes an Increase in IL-1β and IL-13 Protein in Lung Macrophages

To determine if elevated transcripts corresponded with increased cytokine protein expression we examined lung sections of uninfected *Epg5*$^{-/-}$ mice using IHC microscopy. At baseline, F4/80^{+} macrophage-like cells accumulated p62 (Figure 4D), consistent with the known role of *Epg5* in autophagy (Zhao et al., 2013a). Since both M1 and M2 macrophage signatures were identified by RNA-Seq analysis (Figure S3A and S3C) and qPCR (Figure 4A), we co-stained for p62 and IL-1β (produced by M1 macrophages) or IL-13 (produced by M2 macrophages) (Byers et al., 2013; Kim et al., 2008; Wu et al., 2015) in the lung sections of *Epg5* mice. These analyses revealed elevated expression of both cytokines in p62-positive macrophage-like cells in *Epg5*$^{-/-}$ mice (Figure 4E and 4F). These data suggest that macrophages are a source of increased cytokines in *Epg5*$^{-/-}$ mouse lungs.

Deletion of Autophagy Genes in Macrophages Confers Influenza Resistance

Autophagy is impaired in *Epg5* deficient mice (Zhao et al., 2013a). However, *Epg5* also plays a role in endocytic recycling of transferrin receptors and degradation of EGF receptors (Zhao et al., 2013a). Furthermore, *Atg* genes may play roles other than in canonical autophagy (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). Among these non-canonical functions, LAP in macrophages requires *Atg* genes such as *Atg5* and *Atg7*, which have previously been shown to control lung inflammation (Abdel et al., 2015; Kanayama et al., 2015), but does not require *Atg14* or *FIP200* (Martinez et al., 2015). Thus,

prior studies are consistent with a role for either canonical autophagy or LAP in control of lung inflammation (Abdel et al., 2015; Kanayama et al., 2015).

We sought to gain insight into the role of autophagy in macrophages and its consequences for lung inflammation by evaluating the role of additional *Atg* genes deleted specifically in myeloid cells. We deleted loxP-flanked *Atg* genes ($Atg^{flox/flox}$, $Atg^{f/f}$) in LysMcre expressing cells including monocytes, macrophages, and granulocytes (Clausen et al., 1999; Hwang et al., 2012). Mice were challenged with PR8 as before (Figure 5A-E). As observed for $Epg5^{-/-}$ mice, $Atg14^{f/f}$-LysMcre and $FIP200^{f/f}$-LysMcre mice were completely resistant to influenza (Figure 5A-B). As these two genes are not involved in LAP in macrophages (Martinez et al., 2015), it is less likely that a non-canonical function of *Atg* genes is responsible for control of lung inflammation and influenza resistance. Weight loss in $Atg7^{f/f}$-LysMcre and $Atg5^{f/f}$-LysMcre mice was similar to their littermate controls early after infection but recovery was enhanced (Figure 5C-D). Interestingly, $Atg16L1^{f/f}$-LysMcre mice were not resistant to IAV-induced disease (Figure 5E), although we observed p62 accumulation in macrophage-like cells in the lungs of $Atg16L1^{f/f}$-LysMcre mice by IHC (Figure S4A). $Atg4B^{GT/GT}$ mice exhibited a phenotype similar to $Atg16L1^{f/f}$-LysMcre mice (data not shown). These data demonstrated that deficiency of multiple *Atg* genes in myeloid cells, including genes not involved in LAP, is associated with resistance to IAV-induced disease, although the phenotypes vary.

Role of *Atg14, FIP200, Atg5, Atg7*, and *Atg16L1* in Control of Basal Level of Lung Inflammation

Based on similarities to $Epg5^{-/-}$ mice in IAV-resistance, we next evaluated $Atg^{f/f}$-LysMcre mouse lungs for elevated basal inflammation. Lungs of $Atg14^{f/f}$-LysMcre, $FIP200^{f/f}$-LysMcre, $Atg5^{f/f}$-LysMcre, and $Atg7^{f/f}$-LysMcre mice exhibited histologic evidence of increased cellular inflammation and abnormal BAL cells, although lymphoid aggregates were not observed in $Atg5^{f/f}$-LysMcre or $Atg7^{f/f}$-LysMcre mice (Figure 6 and Figure S4B-D). We characterized $Atg14^{f/f}$-LysMcre mice in more detail as they exhibited influenza resistance with kinetics similar to $Epg5^{-/-}$ mice, and exhibited lung lymphoid aggregates (Figure 6A). Abnormal lung histology and BAL cells were observed in $Atg14^{f/f}$-LysMcre mice, as well as increased numbers of total lung cells, AMs, PMNs, and abnormally differentiated AMs (Figure 6A-C). As for $Epg5^{-/-}$ mice (Figure 4), $Atg14^{f/f}$-LysMcre and $FIP200^{f/f}$-LysMcre mice exhibited significant increases in levels of transcripts encoding GM-CSF, TNFα, IL-1β, and MCP-1 (Figure 5F). Changes in cytokine transcripts were less substantial in $Atg5^{f/f}$-LysMcre, $Atg7^{f/f}$-LysMcre, and $Atg16L1^{f/f}$-LysMcre mice. These data indicate that *Atg* genes in myeloid cells regulate both resistance to IAV-induced disease and basal level of cellular and cytokine-related inflammation in the lung.

DISCUSSION

Influenza is a significant pathogen of animals and humans. Here we discovered that *Epg5*, the mouse homolog of a human disease gene, and other *Atg* genes function in macrophages to maintain lung homeostasis by blunting innate immune inflammation. A consequence of this normal homeostatic function is to render mice susceptible to influenza infection. This

reveals that the processes that suppress innate immunity under normal circumstances to limit tissue inflammation can render the host susceptible to viral infection.

We found that the effects of *Epg5* deficiency included: (1) complete protection against lethal influenza and (2) increased innate immune cellular and cytokine-dependent inflammation in the lung prior to IAV challenge. These findings link two sets of previously published data indicating that deletion of the *Atg* genes *Atg5* or *Atg7* results in lung inflammation and that pre-existing lung inflammation can inhibit influenza pathogenesis (Abdel et al., 2015; Ishikawa et al., 2012; Kanayama et al., 2015; Samarasinghe et al., 2014). *Epg5*$^{-/-}$ mice express increased levels of cytokines that have been individually linked to influenza resistance including IL-6, IL-1β, and GM-CSF (Dienz et al., 2012; Huang et al., 2011; Ichinohe et al., 2009; Schmitz et al., 2005; Schneider et al., 2014; Sever-Chroneos et al., 2011; Thomas et al., 2009), suggesting the hypothesis that expression of these cytokine might contribute to influenza resistance.

Our data are most consistent with a role for *Atg* gene function in macrophages as key in the control of lung inflammation and in limiting influenza infection. As the replication of IAV in the mouse model depends on viral replication in epithelial cells, our data indicate that changes in influenza occur through deletion of *Atg* genes in myeloid cells that are not the dominant primary site of viral replication. This suggests that influenza resistance is not due to cell intrinsic effects of *Atg* gene on IAV replication, but is due to effects of *Atg* genes acting *in trans* from macrophages to epithelial cells. This is consistent with a protective role for abnormal expression of cytokines in influenza resistance observed in *Atg* gene-mutant mice.

Tissue Specificity of *Atg* Genes in Regulation of Inflammation

One of the striking findings of this study was the tissue-specificity of elevated cytokine expression in *Epg5*$^{-/-}$ mice. These data suggest that there is an unidentified *Epg5*-regulated process in the lung that limits inflammation and that is less important or absent in other tissues. Alternatively, other tissues may have redundant, non-*Epg5* or *Atg* gene-related processes to control basal innate immune inflammation that are not present in the lung. These data strongly indicate that the effects of *Atg* gene-dependent regulation of inflammation will be specific to individual tissues.

Specificity at the tissue level is reminiscent of the cellular specificity of effects of *Atg* gene deletion in individual immune cell types. For example, deletion of *Atg* genes results in selective death of peripheral rather than thymic T cells and of B1 but not B2 B cells, death of invariant NKT cells, and selective effects on T and B cell memory responses (Chen et al., 2014; Conway et al., 2013; Miller et al., 2008; Pei et al., 2015; Pengo et al., 2013; Pua et al., 2009; Puleston et al., 2014; Stephenson et al., 2009; Xu et al., 2014). In contrast, as shown here, myeloid populations are preserved and even expanded when *Atg* genes are deleted in these cells. The tissue and cell type-specificity observed in these studies indicates that there is no ‘one-size-fits-all’ role for *Atg* genes *in vivo*. Interestingly, we also showed that certain *Atg* genes, functioning in myeloid cells, prevent systemic inflammation induced by chronic herpesvirus infection (Park, et al., 2016). Together these two sets of observations suggest that the systemic application of drugs that inhibit autophagy or *Atg* gene functions for

therapeutic purposes may have highly selective activity for individual tissues and physiologic responses.

Canonical versus Non-canonical Functions of *Atg* Genes *in vivo*

Our studies identified phenotypic variation between mice carrying LoxP-flanked alleles of different *Atg* genes (*Atg14, FIP200, Atg5, Atg7,* and *Atg16L1*) when crossed to a single lineage-specific Cre recombinase-expressing allele. A trivial explanation for phenotypic variations is differential gene deletion due to inconsistent Cre-mediated LoxP site cleavage at different chromosomal loci. While this possibility could contribute to variation, it does not explain all phenotypic differences between *Atg*-LysMcre mouse lines in different biological systems. For example, we reported here that *Epg5*$^{-/-}$, *Atg14*$^{f/f}$-LysMcre, and *FIP200*$^{f/f}$-LysMcre mice exhibit more prominent changes in lung inflammation and influenza resistance than *Atg7*$^{f/f}$-LysMcre, *Atg5*$^{f/f}$-LysMcre, and *Atg16L1*$^{f/f}$-LysMcre mice. In contrast, using the same mouse lines from the same facility, *Atg7*$^{f/f}$-LysMcre, *Atg5*$^{f/f}$-LysMcre, and *Atg16L1*$^{f/f}$-LysMcre mice are all highly susceptible to infection with *Toxoplasma gondii* (*T. gondii*), while *Atg14*$^{f/f}$-LysMcre mice are not (Choi et al., 2014). However, *Atg14*$^{f/f}$-LysMcre, *FIP200*$^{f/f}$-LysMcre, *Atg7*$^{f/f}$-LysMcre, and *Atg5*$^{f/f}$-LysMcre mice exhibit enhanced systemic virus-induced inflammation, limiting murine γ-herpesvirus 68 (MHV68) reactivation from latency (Park *et al.*, 2016). Thus, the lack of a phenotype for *Atg14* in *T. gondii* resistance is likely not due to a failure of Cre-mediated deletion since these same mice exhibited strikingly increased lung inflammation, influenza resistance, and increased control of MHV68.

A further example of the apparent specificity of *Atg* genes for different biological effects is LAP, a process that, at least in macrophages, requires some but not all of the *Atg* genes studied here. Neither *FIP200* nor *Atg14* are required for LAP in macrophages (Martinez et al., 2015) but we found that these genes were essential for maintaining homeostatic control of innate immune activation in the lung. How these biological differences between different mutant mice relate to the cell biological activities of the different genes will be complex to address. These tissue-specific, cell type-specific and biological system-driven effects indicate that a premium should be placed on evaluating the cell biology of these genes in relevant primary cell types.

The lack of a phenotype for *Atg16L1*$^{f/f}$-LysMcre mice was particularly striking because we found that deletion of *Atg16L1* in myeloid cells in the lung resulted in accumulation of p62 and diminished conversion of LC3-I to LC3-II by western blot analysis (Kimmey et al., 2015). Additionally, these same mice are highly susceptible to *T. gondii* infection (Choi et al., 2014) and limit MHV68 reactivation from latency (Park *et al.*, 2016). Perhaps relevant, *ATG16L1* has a homolog, *ATG16L2*, which encodes a ubiquitously-expressed protein that can interact with the ATG5-ATG12 conjugate and with IKKα and therefore may have a role in inflammatory cytokine signaling (Ishibashi et al., 2011; Li et al., 2013). The role of *ATG16L2* in autophagy is controversial, but *ATG16L2* allelic variants are associated with risk for Crohn's disease, supporting its relevance to inflammation (Yang et al., 2014). It will be interesting to examine the role of this gene alone and in combination with mutations in *ATG16L1* in immunity and inflammation especially in the lung.

Potential Roles of *Epg5* in Immunity and Inflammation

In addition to its function in the autophagy pathway, *Epg5* also plays a role in endocytic recycling and degradation (Zhao et al., 2013a). While our studies do not formally delineate the pathway through which *Epg5* controls lung inflammation, the fact that deletion of multiple *Atg* genes in myeloid cells has a similar effect on inflammation supports the hypothesis that *Epg5* plays a role in the lung due, at least in part, to its effects on autophagy. However, many studies have reported connections between the endosomal machinery and autophagy (Knaevelsrud et al., 2013a; Knaevelsrud et al., 2013b; Patel et al., 2013; Razi et al., 2009), indicating that the roles of *Epg5* in these two processes may be related. Interestingly, *Atg14*, which also has a prominent role in control of lung inflammation documented here, has been reported to function in autophagosome formation and endolysosomal fusion (Diao et al., 2015; Itakura et al., 2008; Matsunaga et al., 2009; Sun et al., 2008; Zhong et al., 2009). Together these studies suggest that the studies of intersection between the endosomal trafficking and autophagy may be of particular interest to define mechanisms of immune cell function.

Epg5$^{-/-}$ mice and Vici Syndrome

Vici syndrome patients suffer from recurrent pulmonary infections (Ehmke et al., 2014). However, our study shows that the *Epg5* deficient mice are resistant to influenza. The reasons for this apparent inconsistency are uncertain, but may be related to the following considerations. First, IAV is notably absent from the list of pathogens responsible for recurrent pulmonary infections in Vici syndrome patients (Ehmke et al., 2014; Finocchi et al., 2012). Thus, it is possible that Vici syndrome patients are resistant to influenza, yet more susceptible to other forms of pulmonary infection. Second, recurrent pulmonary infections in Vici syndrome patients may occur secondary to neuropathic and tracheal myopathic abnormalities (Ehmke et al., 2014), and such effects may be dominant over changes in baseline lung inflammation. The relationship of the findings reported here to the clinical situation remains to be determined.

EXPERIMENTAL PROCEDURES

Mice and Cells

Crosses of *Epg5*$^{+/-}$ (Zhao et al., 2013a) mice were used to generate experimental animals and littermates were used for all experiments. *Rag1*$^{-/-}$ (Jackson Laboratory) mice were bred to *Epg5*$^{-/-}$ mice. CD45.1 (B6.SJL-PtprcaPepcb/BoyJ) mice were from Jackson Laboratory. All *Atg* mice were previously described. All mice were housed and bred at Washington University in Saint Louis in specific pathogen-free conditions in accordance with federal and university guidelines as previously described (Cadwell et al., 2008). Generation of murine MEFs and BMDMs was as described (Cadwell et al., 2008; Hwang et al., 2012). Madin-Darby canine kidney (MDCK) cells were used for influenza A virus titration. See Supplemental Experimental Procedures for the details of *Atg* mice.

Inoculation of Mice with IAV and Determination of Lung Viral Titers

H1N1 IAV strains used: A/California/04/2009 (California) and A/Puerto Rico/8/1934 (PR8). 6-8 week mice were infected with 10^4 EID_{50} (50% egg infectious dose) of California or 50 $TCID_{50}$ (50% tissue culture infectious dose) of PR8 intranasally and morbidity and mortality were monitored. Mice losing more than 30% of their initial body weight were sacrificed. To determine pulmonary viral titers, mice were sacrificed 2 dpi. Lungs were harvested and homogenized and virus was titrated by $TCID_{50}$. See Supplemental Experimental Procedures for the details of $TCID_{50}$ titration.

Lung Histological Analysis and Immunohistochemistry

Paraffin embedded lung sections were stained with H&E or PAS (Periodic Acid–Schiff). For IHC staining, lung sections were deparaffinized, incubated with primary antibodies and detected by fluorescence-conjugated secondary antibodies. See Supplemental Experimental Procedures for details.

Evaluation of BAL

Mice were sacrificed and a catheter (SR-OX2225CA, Terumo) was inserted into the trachea. The lungs were lavaged and collected cells were treated with ACK lysis buffer (Lonza), and then adhered to a slide by centrifugation. Wright-Giemsa staining was performed using HEMA 3 Stat Pack (Protocol, Fisher).

Flow Cytometric Analysis of Lung Cells

Single-cell suspensions were made from lung tissues, blocked with 2.4G2 (anti-FcγRII/III, Biolegend), then labeled with specific antibodies. Flow cytometric analysis was performed on an LSRFortessa (BD Biosciences) and data analyzed with FlowJo software (Tree Star Inc.). See Supplemental Experimental Procedures for anitbodies, gating strategy of cell populations and data analysis.

RNA Analysis

Tissue samples were homogenized with 1.0 mm silica beads on the mini BeadBeater (Biospec) and RNA was extracted using Trizol (Ambion) followed by cDNA synthesis with the ImPromII reverse transcriptase system (Promega). qPCR was performed using SYBR green master mix (Life Technologies) or AmpliTaq 360 (Life Technologies). Transcript levels were normalized to the level of *Actin* within each sample and compared to the level in control mice using the $\Delta\Delta Ct$ method. Primers used are shown in Supplemental Table 2.

Statistical Analysis

Data were analyzed with Prism 6 software (GraphPad Software, San Diego, CA). Mann Whitney test was performed to compare two groups, one-way ANOVA (Tukey post test) multiple comparisons were used to compare three or more groups. Differences in survival were determined using a log-rank test with Bonferroni correction for multiple comparisons. All differences not specifically stated to be significant were not significant ($P>0.05$). For all figures, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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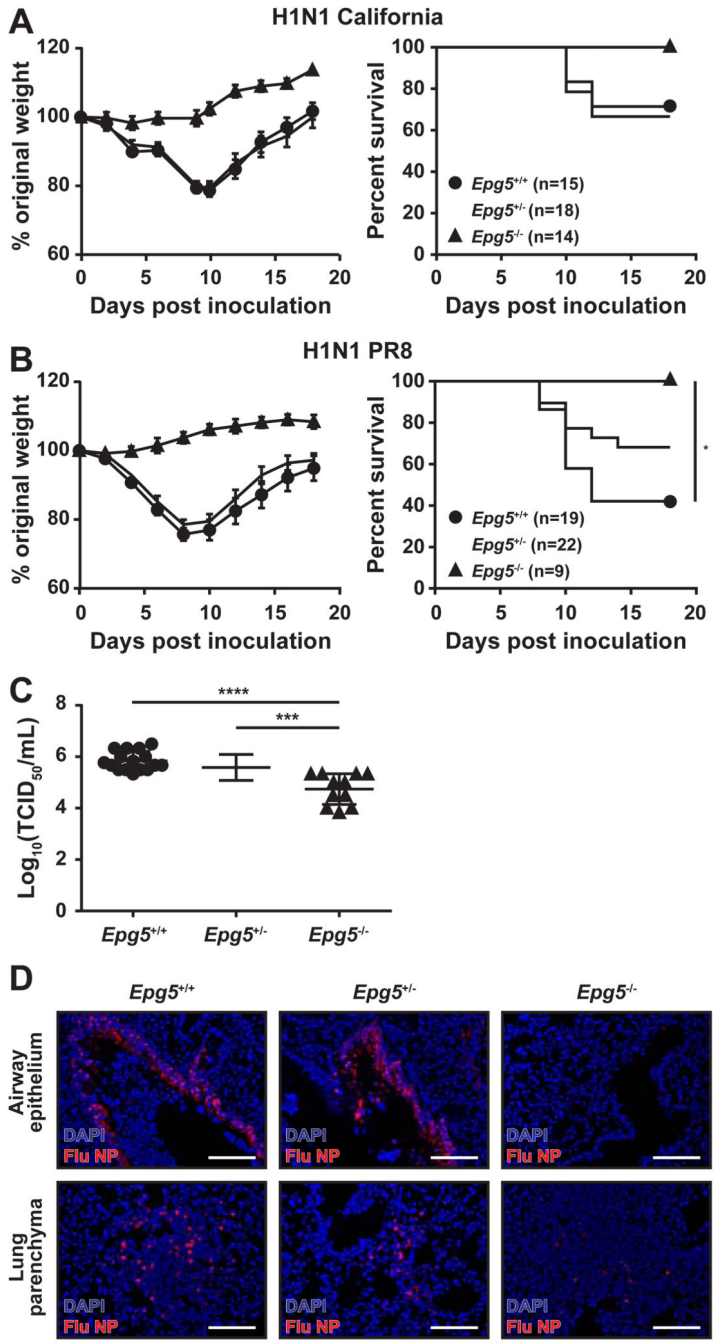

Figure 1. *Epg5* Deficiency Protects Mice from Influenza

*Epg5*mice were infected with 10^4 EID_{50} California (A) or 50 $TCID_{50}$ PR8 (B) and monitored for weight loss and mortality.

(C) Mice were infected with 50 $TCID_{50}$ PR8. At 2 days post infection (dpi), viral titers were determined by $TCID_{50}$. Each symbol represents an individual mouse and the mean $Log_{10}TCID_{50}$/ml ± SEM is indicated.

(D) Mice were infected with 50 $TCID_{50}$ PR8. At 2 dpi, lungs were harvested, sectioned, and stained for flu-NP with DAPI counterstaining. Representative photomicrographs are shown (n= 4~8). Scale bars 100μm.

See also Figure S1.

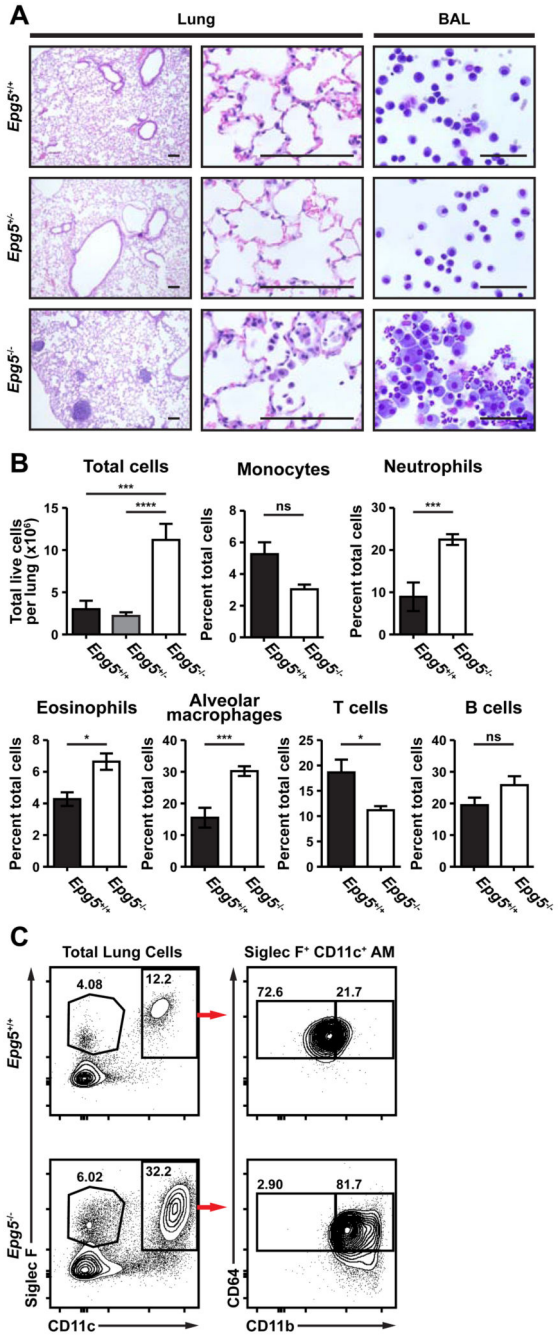

Figure 2. Cellular Lung Inflammation in *Epg5* Deficiency Micew

(A) Lung sections from 8 week-old naive mice H&E stained. BAL cells were adhered to slides and subjected to Wright-Giemsa staining.

(B) Single cell suspensions were prepared from naïve adult *Epg5* mouse lungs and evaluated by flow cytometry, with pre-gating on $CD45^+$ cells.

(C) Flow cytometry plots, representative of 9-13 mice per genotype across 3 independent experiments. $SiglecF^+CD11c^+$ AM populations were further analyzed for CD64 and CD11b expression. Data represent the mean ± SEM. Scale bars 100μm.

See also Figure S2.

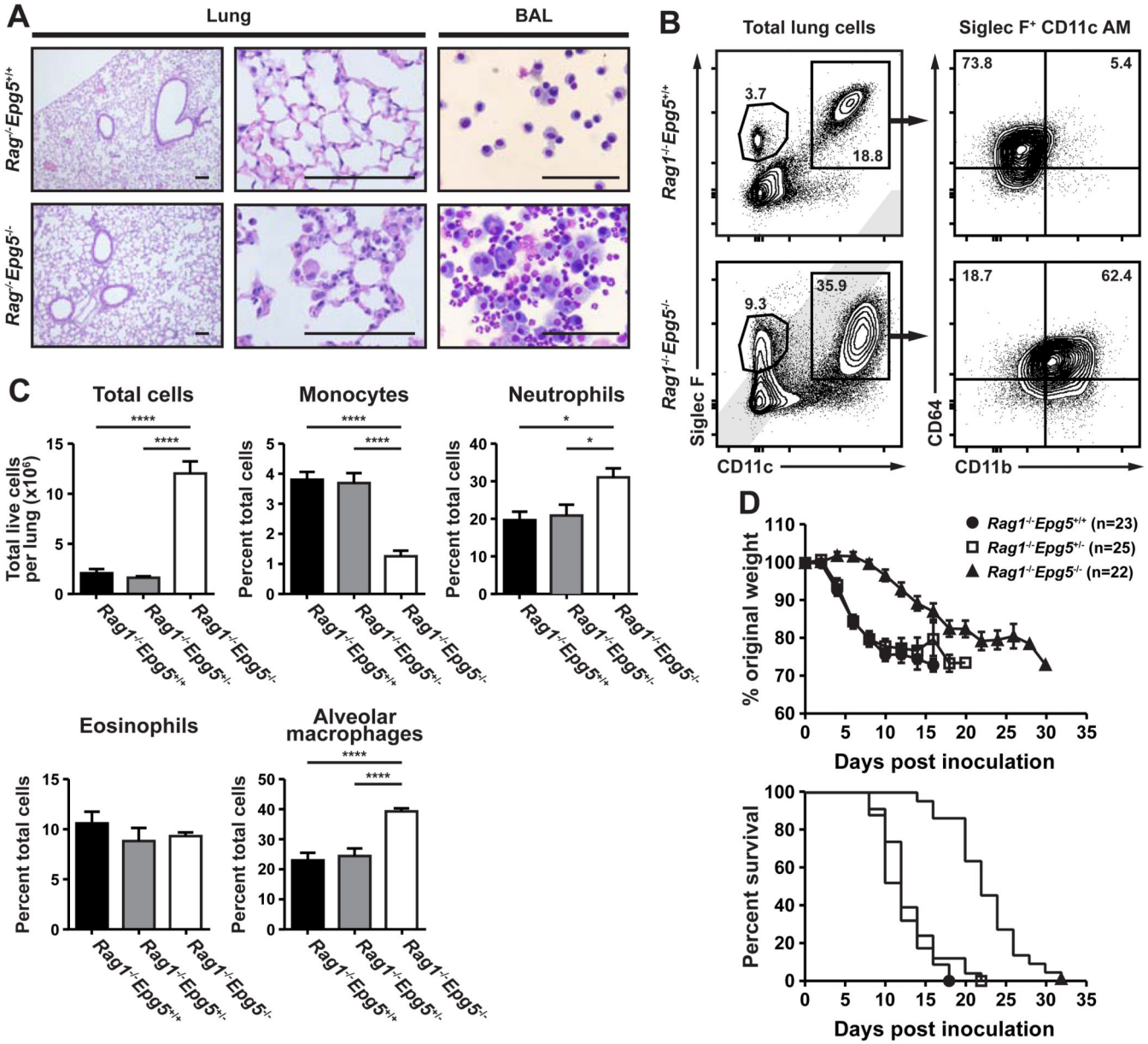

Figure 3. *Epg5* Deficiency Enhances Innate Immunity to Influenza

(A) Lung sections and BAL from 8 week-old naïve *Rag1*$^{-/-}$*Epg5* mice. Scale bars 100μm.

(B) Single cell suspensions were prepared from naïve adult *Rag1*$^{-/-}$*Epg5* mouse lungs and analyzed by flow cytometry. Representative plots are shown.

(C) Flow cytometry analysis of total cell number and cell populations in the lungs of *Rag1*$^{-/-}$*Epg5* mice. (B-C) Data represent the mean ± SEM of 8-11 mice per genotype across at least three independent experiments.

(D) *Rag1*$^{-/-}$*Epg5* mice were infected with 50 $TCID_{50}$ PR8 and monitored for weight loss and mortality.

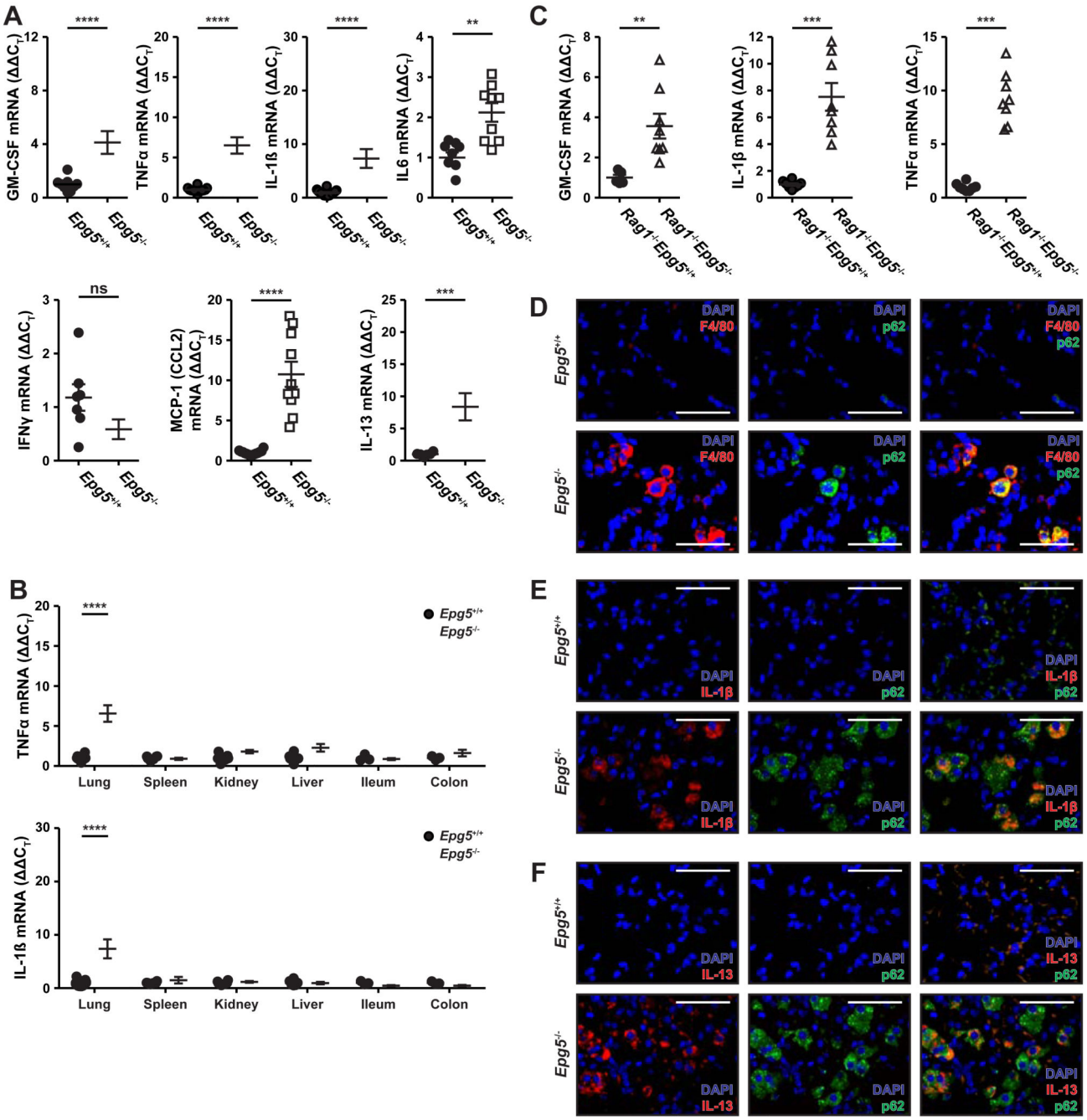

Figure 4. *Epg5* Regulates Basal Expression of Cytokines Important for Control of Influenza in the Lung

RNA was prepared from the (A) lungs or (B) indicated organs of *Epg5* mice and the indicated transcripts were determined by qPCR.

(C) RNA was prepared from the lungs of *Rag1*$^{-/-}$*Epg5* mice and the indicated transcripts were evaluated by qPCR. Each symbol represents an individual mouse. Data represent the mean ± SEM.

(D-F) Lung sections from *Epg5* mice were stained for p62 in combination with F4/80 (D), IL-1β (E) or IL-13 (F). Scale bars 50μm.

See also Figure S3.

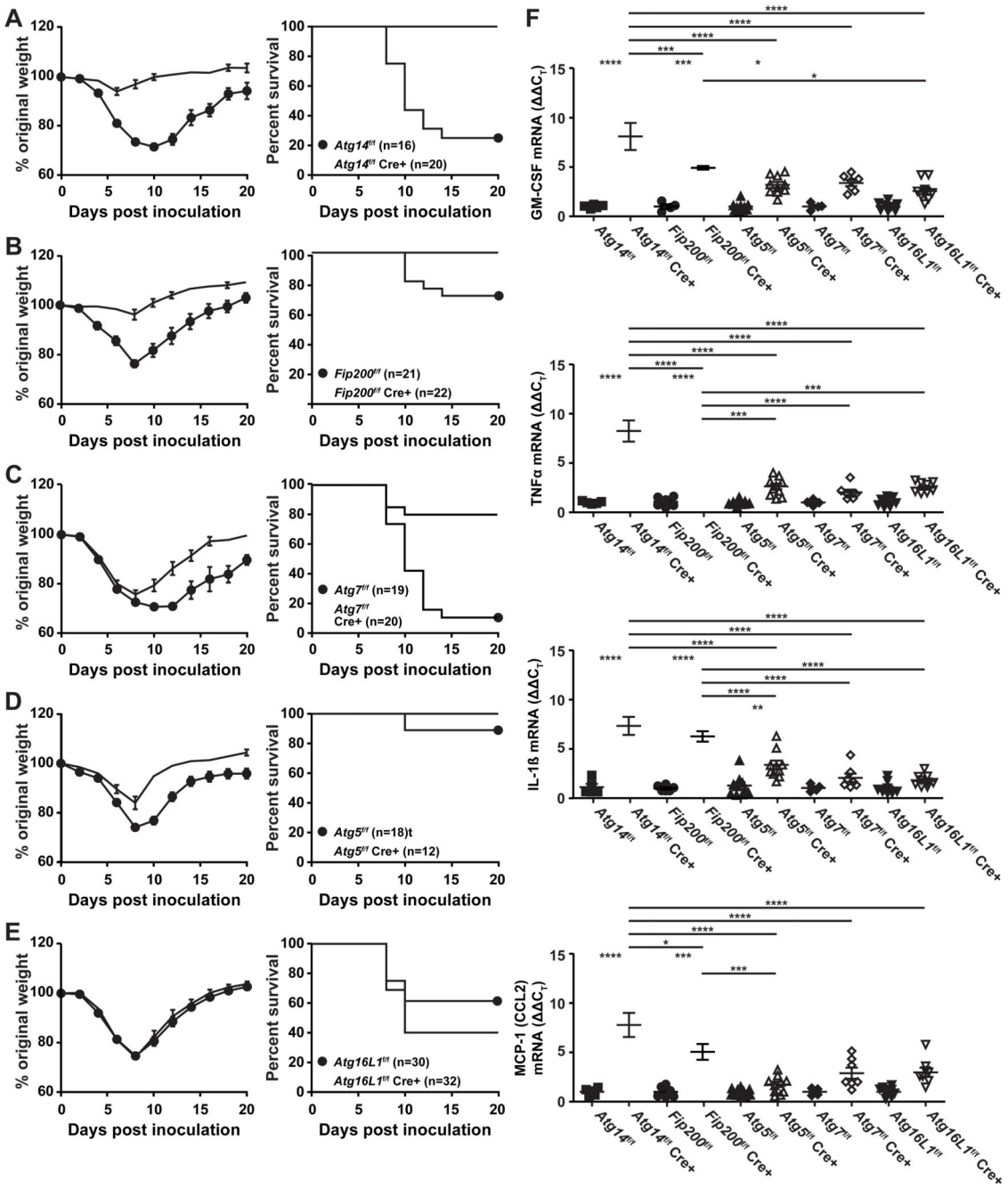

Figure 5. Deletion of Autophagy Genes in Myeloid Cells Causes Resistance to Influenza and Basal Hyper-inflammation in the Lung

(A-E) Survival and weight loss of *Atg14*$^{f/f}$-LysMcre (A), *FIP200*$^{f/f}$-LysMcre (B), *Atg7*$^{f/f}$-LysMcre (C), *Atg5*$^{f/f}$-LysMcre (D) and *Atg16L1*$^{f/f}$-LysMcre (E) mice following inoculation with 50 $TCID_{50}$ PR8.

(F) GM-CSF, TNFα, IL-1β and MCP-1 transcript levels were measured by qPCR in the lungs of the indicated mouse genotypes. Each symbol represents an individual mouse. Data represent the mean ± SEM.

See also Figure S4.

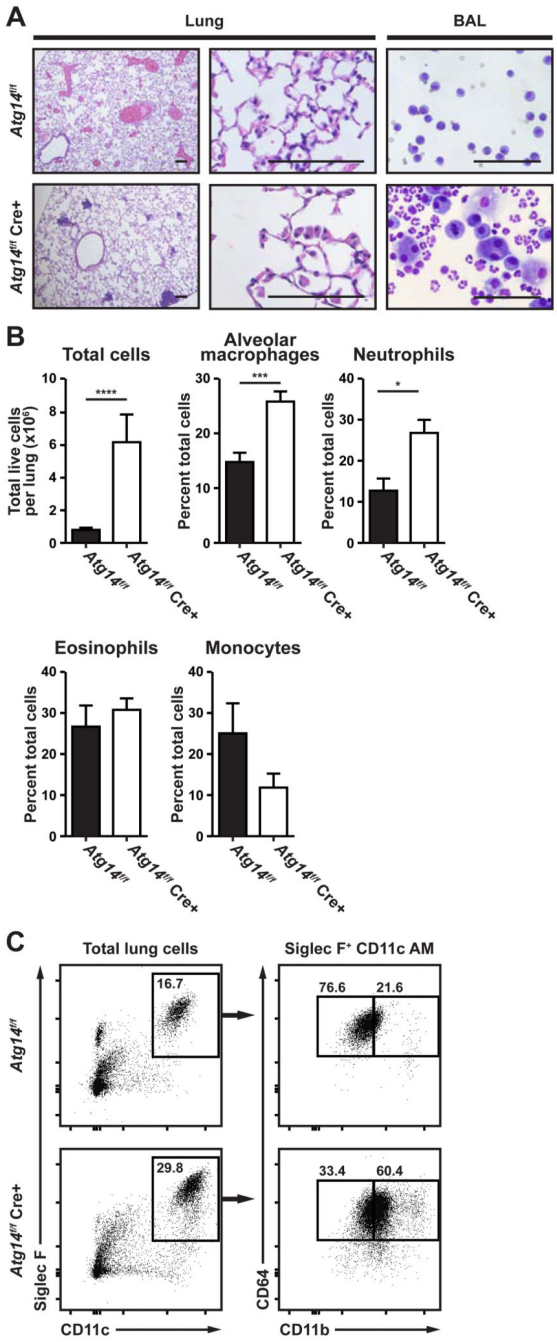

Figure 6. Cellular Lung Inflammation in *Atg14*$^{f/f}$-LysMcre Mice

(A) 8-10 week-old naïve *Atg14*$^{f/f}$-LysMcre mice were analyzed for lung histology and BAL content. Scale bars 100μm.

(B) Quantitation of cell number and populations in *Atg14*$^{f/f}$-LysMcre mice.

(C) Flow cytometry analysis of AMs in *Atg14*$^{f/f}$-LysMcre mice. Data are representative of three independent experiments with 9-10 mice in each genotype.