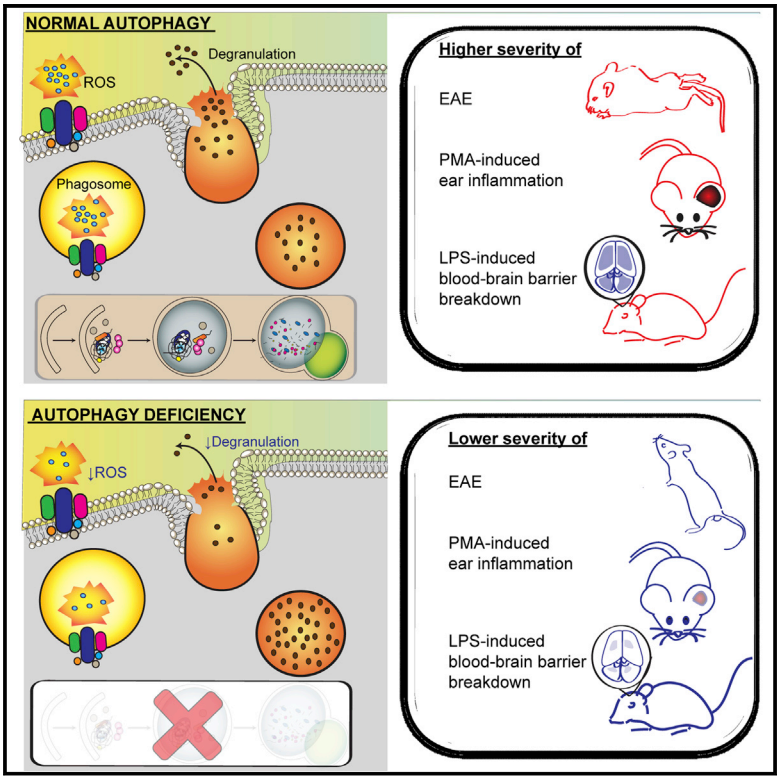
Report

Cell Reports

Autophagy Is Required for Neutrophil-Mediated Inflammation

Graphical Abstract



Highlights

- Autophagy regulates neutrophil degranulation
- Autophagy regulates NADPH-oxidase-mediated reactive oxygen species production
- Autophagy deficiency reduces inflammatory activity of neutrophils

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In Brief

Using several mouse models, Bhattacharya et al. identify autophagy as an important regulator of neutrophil degranulation and ROS production by NADPH oxidase. The authors find that autophagy is required for inflammatory activity of neutrophils.

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Cell Reports
Report

Autophagy Is Required for Neutrophil-Mediated Inflammation

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SUMMARY

Autophagy, an intracellular degradation and energy recycling mechanism, is emerging as an important regulator of immune responses. However, the role of autophagy in regulating neutrophil functions is not known. We investigated neutrophil biology using myeloid-specific autophagy-deficient mice and found that autophagy deficiency reduced neutrophil degranulation in vitro and in vivo. Mice with autophagy deficiency showed reduced severity of several neutrophil-mediated inflammatory and autoimmune disease models, including PMA-induced ear inflammation, LPS-induced breakdown of blood-brain barrier, and experimental autoimmune encephalomyelitis. NADPH oxidase-mediated reactive oxygen species generation was also reduced in autophagy-deficient neutrophils, and inhibition of NADPH oxidase reduced neutrophil degranulation, suggesting NADPH oxidase to be a player at the intersection of autophagy and degranulation. Overall, this study establishes autophagy as an important regulator of neutrophil functions and neutrophil-mediated inflammation in vivo.

INTRODUCTION

Neutrophils play a critical role as one of the first lines of innate immune defense. Activated neutrophils migrate to the site of inflammation and control microbes by phagocytosis, formation of neutrophil extracellular traps, and secretion of antimicrobials, stored in specialized granular compartments. Neutrophils possess at least four types of granules. Among these, primary, secondary, and tertiary granules develop from the endoplasmic reticulum (ER)-Golgi network, whereas secretory vesicles have an endocytic origin (Borregaard, 1997). Primary (azurophilic) granules contain myeloperoxidase (MPO), β-glucuronidase, elastase, and other antimicrobial factors, whereas lactoferrin and matrix metalloprotease-9 (MMP-9) are stored in secondary (specific) granules. Tertiary (gelatinase) granules also contain MMP-9 but lack lactoferrin. The membranes of secretory vesicles contain several important molecules, including β2-integrins (CD11b/CD18) and complement receptors, which play an essential role in neutrophil migration and activation (Amulic et al., 2012). Upon activation, these granules are mobilized, fuse with phagosomes or plasma membrane, and release their contents in the respective environments. Secondary granules are of interest, as the membranes of these granules contain flavocytochrome b_{558}, a component of the NADPH oxidase machinery (Amulic et al., 2012). Thus, fusion of secondary granules with phagosomes or plasma membrane results in formation of a functional NADPH oxidase complex, which then produces reactive oxygen species (ROS). Degranulation, a process of regulated exocytosis of granules, is one of the major mechanisms of inflammatory response by neutrophils. However, the underlying regulatory pathways and their respective roles remain incompletely understood.

Autophagy plays important roles in the immune responses and abnormalities in this pathway have been linked to several diseases (Levine et al., 2011). More recently, autophagy has been implicated in regulating secretion (Cadwell et al., 2008; Patel et al., 2013; Ushio et al., 2011; Bhattacharya et al., 2014b), though the underlying mechanisms and in vivo relevance are not fully understood. The role of autophagy in neutrophil functions remains largely unexplored.

Here, we show that deficiency of autophagy reduced degranulation of neutrophils. Autophagy-deficient neutrophils had reduced NADPH oxidase-mediated ROS generation. Moreover, inhibition of NADPH oxidase reduced neutrophil degranulation, implicating NADPH oxidase in mediating effects of autophagy on neutrophil degranulation. The in vivo relevance of these findings was shown in the context of autoimmune and inflammatory processes. These findings establish autophagy as an important regulator of neutrophil functions and suggest that targeting autophagy pathway could have therapeutic value during infection, inflammation, or neutrophil-mediated autoimmune diseases.

RESULTS

Deficiency of Autophagy Reduced Neutrophil Degranulation

To evaluate the role of autophagy in neutrophils, we generated mice with myeloid-specific deletion of *Atg7* (called $Atg7^{M\Delta}$ mice hereafter) by breeding mice bearing floxed *Atg7* alleles ($Atg7^{f/f}$

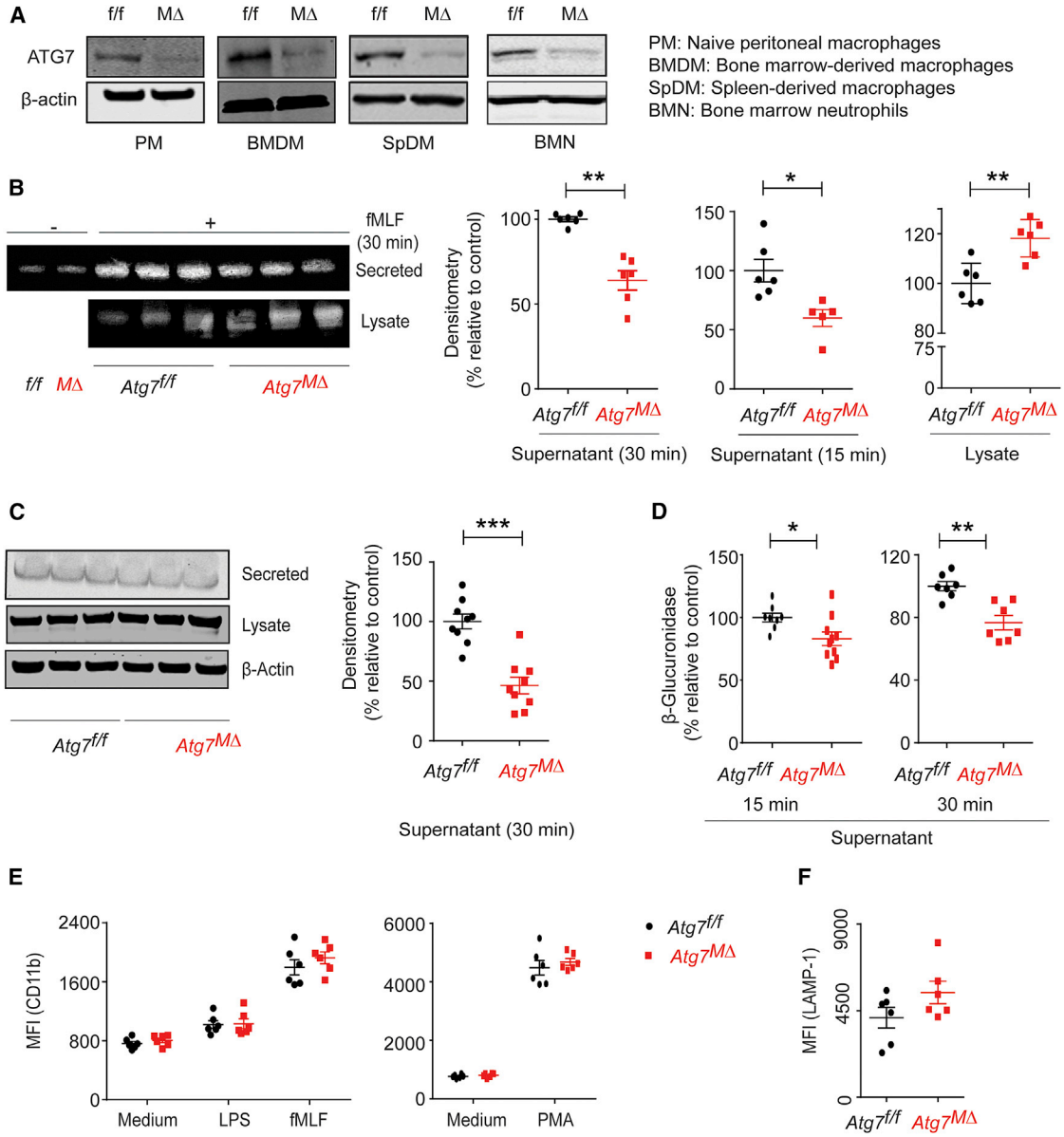

Figure 1. ATG7 Deficiency Reduced Neutrophil Degranulation

(A) Representative immunoblot of ATG7 using lysates from purified peritoneal macrophages (PM), bone marrow-derived macrophages (BMDM), spleen-derived macrophages (SpDM), and bone marrow neutrophils (BMN). Lanes marked f/f and MΔ denote lysates from control and *Atg7*$^{M\Delta}$ mice, respectively.

(B) BMN from control or *Atg7*$^{M\Delta}$ mice were primed with cytochalasin B and then stimulated with 1 μM fMLF for 15 or 30 min. Gelatinase zymography was performed on supernatants and on lysates from unstimulated BMN; n = 6/group. Densitometry analysis and a representative image, in which each lane or symbol represents one mouse, are shown.

(C) Lysates from unstimulated BMN, and supernatant collected following cytochalasin B and 1 μM fMLF treatment of *Atg7*$^{f/f}$ and *Atg7*$^{M\Delta}$ neutrophils were used for immunoblotting by lactoferrin antibody. n = 6–9/group. Each lane or symbol represents one mouse.

(D) BMN from control or *Atg7*$^{M\Delta}$ mice were stimulated as above. Supernatant was collected and incubated overnight with p-nitrophenyl-β-D-glucuronide (for β-D-glucuronidase). OD values were measured at 405 nm.

(E and F) BMN were stimulated with cytochalasin B and 1 μM fMLF, 1 μg/ml LPS, or 1 μg/ml PMA for 15 min and evaluated by flow cytometry using antibodies against Ly6G and CD11b (E) or LAMP-1 (F). n = 6/group. Each symbol represents one mouse.

Data are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. MFI, mean fluorescence intensity.

mice) with transgenic LysM-cre mice (Abdel Fattah et al., 2015; Bonilla et al., 2013; Shin et al., 2013). Bone marrow neutrophils (BMNs) from *Atg7*$^{M\Delta}$ mice showed substantial reduction of ATG7, and the efficiency of genetic deletion was comparable to that observed in peritoneal macrophages or macrophages generated from bone marrow or spleen from *Atg7*$^{M\Delta}$ mice (Figure 1A).

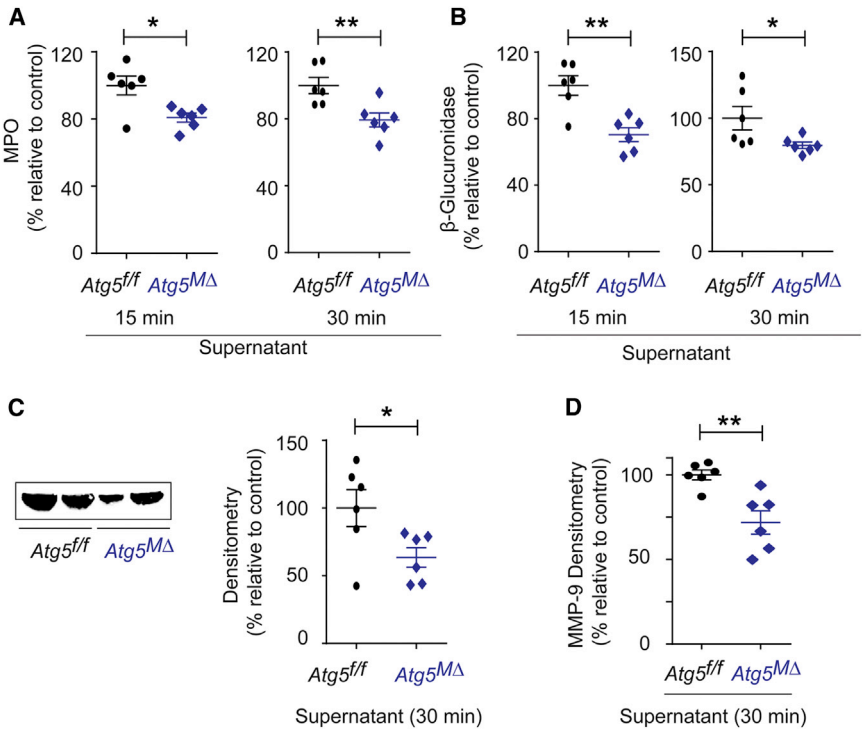

Figure 2. ATG5 Deficiency Reduced Neutrophil Degranulation

BMN from control or $Atg5^{M\Delta}$ mice were primed with cytochalasin B and then stimulated with 1 μM fMLF for 15 or 30 min.

(A and B) Supernatant was collected, incubated for 15 min with tetramethyl-benzidine to determine levels of MPO (A), or incubated overnight with p-nitrophenyl-β-D-glucuronide to determine β-D-glucuronidase (B).

(C) In addition, supernatant was used for immunoblotting by lactoferrin antibody. One representative blot in which each lane corresponds to one mouse and densitometry analysis is shown.

(D) Gelatinase zymography was performed on supernatant, and densitometry analysis is shown. For all panels, each symbol represents one mouse. n = 6/group. Data are mean ± SEM; *p < 0.05, **p < 0.01.

As autophagy has been recently implicated in the secretory pathway, we hypothesized that autophagy deficiency would affect neutrophil degranulation. Among the granules originating from ER-Golgi apparatus, tertiary granules are the first to release their contents upon activation. To test the effect of autophagy on degranulation of tertiary granules, we stimulated BMN with cytochalasin B and bacterial tripeptide fMLF, and used the supernatant for gelatinase zymography. Loss of ATG7 reduced the levels of MMP-9 secreted after fMLF treatment (Figure 1B). Gelatinase zymography, performed on the lysates of unstimulated BMN, showed that baseline levels of MMP-9 in $Atg7^{M\Delta}$ neutrophils were paradoxically elevated (Figure 1B), suggesting that the reduction in MMP-9 secretion was due to retention, rather than a defect in its synthesis.

To determine whether autophagy was involved in the secretion of other types of neutrophil granules, we stimulated BMN with cytochalasin B and fMLF and evaluated the secretion of β-D-glucuronidase (stored in primary granules) and lactoferrin (stored in secondary granules). Deficiency of ATG7 reduced degranulation from both primary and secondary granules (Figures 1C and 1D). In contrast, upregulation of CD11b in response to various stimuli remained unaltered in $Atg7^{M\Delta}$ neutrophils (Figure 1E). Secretory lysosomes were also unaffected, as evidenced by similar LAMP-1 levels in ATG7-deficient and control neutrophils following PMA stimulation (Figure 1F).

To rule out autophagy-independent roles of *Atg7* in neutrophil degranulation, we generated mice with myeloid cell-specific deletion of *Atg5* ($Atg5^{M\Delta}$ mice) (Abdel Fattah et al., 2015). We found that deletion of *Atg5* reduced degranulation of primary, secondary, and tertiary granules from BMNs (Figure 2). These data suggested that autophagy was required for neutrophil degranulation.

We then wanted to determine whether there was a global dysfunction in autophagy-deficient neutrophils. We have previously shown that zymosan phagocytosis was not altered in autophagy-deficient macrophages (Bonilla et al., 2013). To test whether autophagy deficiency altered zymosan uptake by neutrophils, we incubated BMN from control or $Atg7^{M\Delta}$ mice with FITC-labeled zymosan A and checked phagocytosis by flow cytometry. There was no significant difference in zymosan A uptake, or surface levels of CD11b, between control and $Atg7^{M\Delta}$ neutrophils (Figures S1A and S1B). However, consistent with our previous findings, we observed substantial reduction of MMP-9 in the supernatant collected after incubation of zymosan A with $Atg7^{M\Delta}$ neutrophils (Figure S1C). Thus, the defect in neutrophil degranulation in $Atg7^{M\Delta}$ mice was not part of a global dysfunction in these cells.

Reduced In Vivo Neutrophil-Mediated Inflammation in $Atg7^{M\Delta}$ Mice

We hypothesized that autophagy deficiency would reduce severity of neutrophil-mediated inflammatory diseases in vivo. To test this hypothesis, we utilized a model of neutrophil-mediated PMA-induced ear inflammation (Gross et al., 2009). $Atg7^{M\Delta}$ mice showed substantial reduction in ear swelling (Figure 3A). After PMA application, degranulation of MPO, detectable by in vivo bioluminescence imaging, requires fusion of primary granules with phagosomes (Gross et al., 2009). Neutrophil MPO in $Atg7^{M\Delta}$ mice was substantially reduced (Figure 3B). These data suggested that autophagy deficiency reduced degranulation of primary granules by reducing their fusion with phagosomes.

We then extended our findings to another in vivo model of inflammation. Intraperitoneal LPS injection increases the permeability of blood-brain barrier, an effect dependent on MMP-9 (Aid

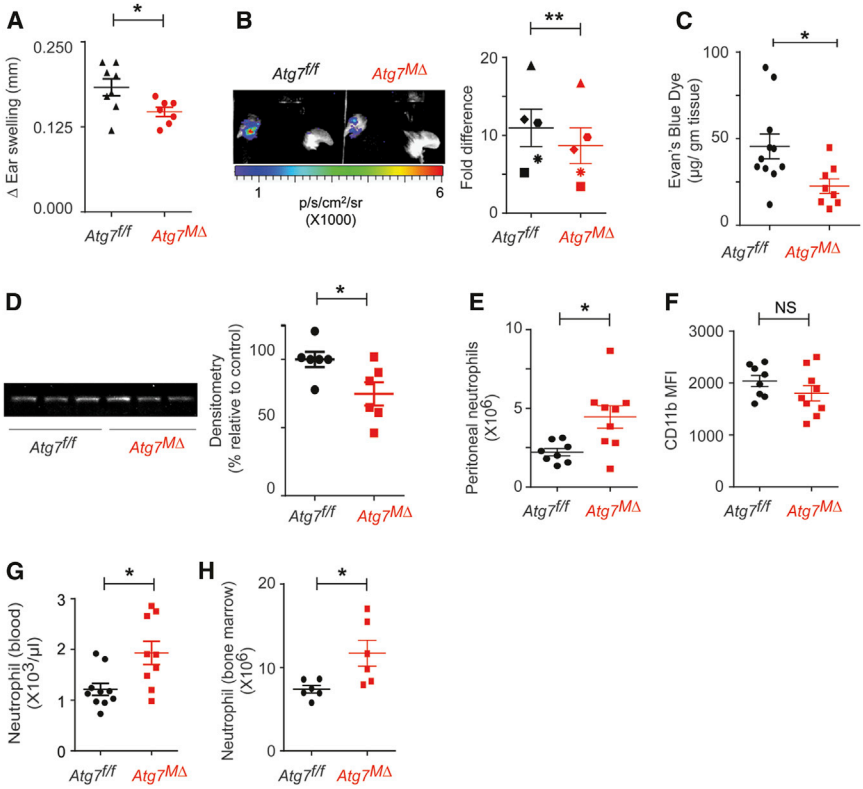

Figure 3. Reduced Neutrophil-Mediated Inflammation in *Atg7*$^{M\Delta}$ Mice In Vivo

(A and B) Ear inflammation was induced by topical application of PMA for 24 hr, and ear thickness was then measured; n = 7–8/group (A). Bioluminescence imaging was performed. A representative image and quantitative analysis are shown (B). Similar symbols denote matched pairs. n = 7/group.

(C) LPS was injected intra-peritoneally (5 mg/kg, at 0 and 48 hr), and blood-brain barrier permeability was measured.

(D) BMN were isolated from control and *Atg7*$^{M\Delta}$ mice and stimulated with 1 μg/ml LPS for 30 min, and supernatant was subjected to gelatinase zymography. A representative zymogram, in which each lane denotes one mouse, and quantitative analyses are shown. n = 6/group.

(E and F) Peritoneal exudate cells were collected 4 hr after thioglycollate injection. Neutrophils were isolated and stained with Ly6G (E) and CD11b (F).

(G) Blood was collected by cardiac puncture, and complete blood count was performed.

(H) BMN were isolated and counted. For all panels, each symbol represents one mouse.

Data are mean ± SEM; *p < 0.05, **p < 0.01. MFI, mean fluorescence intensity.

et al., 2010; Bennett et al., 2010). After LPS administration, we observed that *Atg7*$^{M\Delta}$ mice showed substantially reduced permeability of the blood-brain barrier (Figure 3C). Moreover, MMP-9 release, following stimulation of BMN with LPS, was also reduced from autophagy-deficient neutrophils (Figure 3D). These data suggested that autophagy-deficiency reduced overall inflammatory potential of neutrophils.

Increased Circulating Neutrophils in *Atg7*$^{M\Delta}$ Mice

To rule out defects in neutrophil migration, we used a peritonitis model in which peritoneal exudate cells were collected 4 hr after intraperitoneal thioglycollate injection. Surprisingly, peritoneal neutrophils were increased in *Atg7*$^{M\Delta}$ mice, though no differences in CD11b levels were observed in these neutrophils (Figures 3E and 3F). In vitro transwell migration assay showed no gross defect in *Atg7*$^{M\Delta}$ neutrophil migration in response to different stimuli (Figure S1D). Complete blood analysis of *Atg7*$^{M\Delta}$ mice and their littermate controls showed similar total number of white blood cells (Figure S2), but total and differential neutrophil counts were higher in the *Atg7*$^{M\Delta}$ mice (Figures 3G and S2). The increase in circulating neutrophils in *Atg7*$^{M\Delta}$ mice was likely the result of increased neutrophil content in the bone marrow of *Atg7*$^{M\Delta}$ mice (Figure 3H). These data suggested that myeloid-specific autophagy deficiency increased the number of circulating neutrophils and, consequently, their recruitment to the site of inflammation. However, because of their reduced effector functions, these neutrophils could not efficiently mediate inflammation.

Autophagy Deficiency in Myeloid Cells Reduced Severity of Experimental Autoimmune Encephalomyelitis

To further test the inflammatory potential of autophagy-deficient neutrophils, we used experimental autoimmune encephalomyelitis (EAE), an autoimmune model, which mimics several aspects of multiple sclerosis disease in humans. Neutrophils have been implicated as a critical mediator of CNS inflammation during the effector phase of EAE and neutrophil depletion at different phases of EAE inhibits or abolishes EAE (McColl et al., 1998; Rumble et al., 2015; Steinbach et al., 2013). Secretion of proteases, such as MMP-9, by neutrophils facilitates breakdown of blood-CNS barrier during EAE (Opdenakker et al., 2001). We found that, after EAE induction by active immunization, both control and *Atg7*$^{M\Delta}$ mice showed 100% incidence of EAE. However, the severity of EAE was lower in *Atg7*$^{M\Delta}$ mice (Figure 4A). Additionally, weight loss and cumulative disease score (area under the curve) were attenuated in *Atg7*$^{M\Delta}$ mice (Figures 4B and 4C).

Consistent with the roles of MMP-9 in breakdown of blood-CNS barrier, we observed an increase in MMP-9 in spinal cord lysates, correlating with severity of EAE (Figure S3A). Previous reports showed that blood-spinal cord barrier, rather than the blood-brain barrier, was predominantly affected in mouse model of EAE (Bennett et al., 2010). At the peak of EAE, we observed reduced amount of Evan's blue dye in the spinal cord, but not in organs without permeability barrier, in *Atg7*$^{M\Delta}$ mice (Figures 4D and S3B). Consistent with attenuation of breakdown of

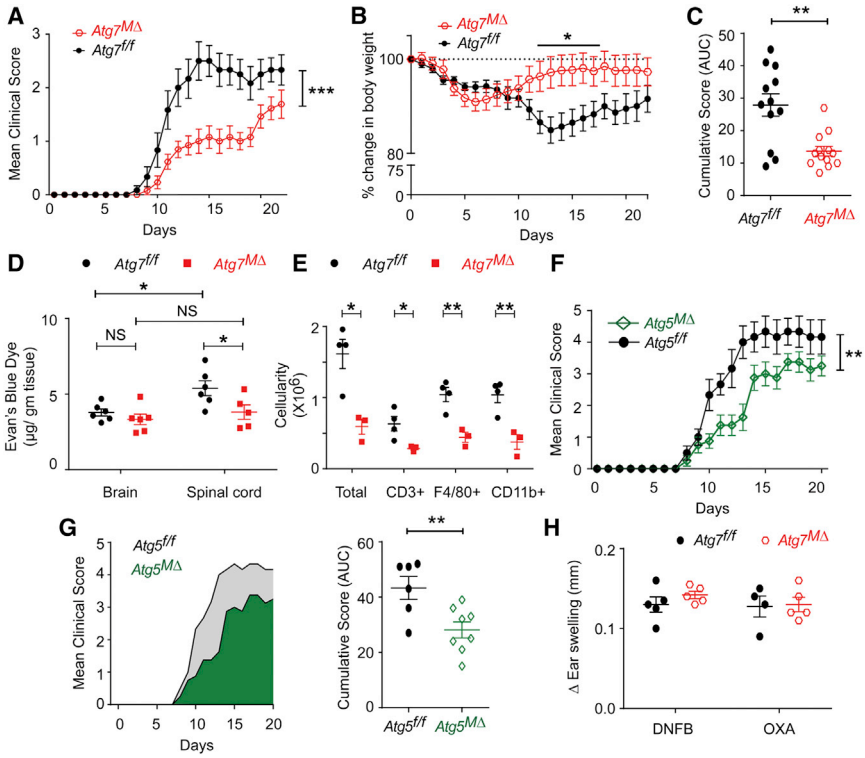

Figure 4. Autophagy Deficiency in Myeloid Cells Reduced Severity of EAE

(A–C) EAE was induced in *Atg7*$^{M\Delta}$ mice and littermate controls, and daily clinical scores (A), daily weight changes (B), and cumulative disease scores (C) were recorded. n = 12–13/group.

(D) Evaluation of brain and spinal cord barrier permeability at day 16 after EAE induction.

(E) Mice were sacrificed at day 16 after EAE induction, and CNS-infiltrating cells were isolated by Percoll gradient.

(F and G) EAE was induced in *Atg5*$^{M\Delta}$ mice and littermate controls, and daily clinical scores (F) and cumulative disease scores (G) were monitored. n = 10–12/group.

(H) Contact hypersensitivity was induced in *Atg7*$^{M\Delta}$ and control mice by sensitization and challenge with Oxazolone (OXA) or DNFB. Ear swelling was measured 24 hr after challenge. Each symbol represents one mouse. AUC, area under the curve.

Data are mean ± SEM; *p < 0.05, **p < 0.01.

blood-CNS barrier permeability, we found a significant reduction in inflammatory cells in the CNS of the *Atg7*$^{M\Delta}$ mice at the peak of EAE (Figure 4E). *Atg5*$^{M\Delta}$ mice also showed a significant reduction in severity and cumulative disease score of EAE compared to their littermate controls (Figures 4F and 4G). Six out of ten *Atg5*$^{f/f}$ mice reached score 5 compared to one out of 12 *Atg5*$^{M\Delta}$ mice (*p = 0.02, chi-square test). Thus, deficiency of either *Atg5* or *Atg7* in myeloid cells resulted in attenuated EAE phenotype.

Finally, to rule out global immunosuppression in these mice, we utilized models of hapten-induced contact hypersensitivity (CHS). CHS is mediated primarily by CD8 T cells (Vocanson et al., 2009). The severity of hapten-induced CHS in response to Oxazolone or DNFB remained undiminished in *Atg7*$^{M\Delta}$ mice (Figure 4H). Thus, the reduction in severity of EAE in *Atg7*$^{M\Delta}$ mice was not due to a global reduction in immune functions.

Peripheral Antigen-Specific T Cell Generation in EAE Was Unaltered in *Atg7*$^{M\Delta}$ Mice

To rule out reduced peripheral T cell activation in *Atg7*$^{M\Delta}$ mice, we examined MOG-specific T cell proliferation at different phases of EAE. There was no significant reduction in antigen-specific T cell proliferation in spleen of *Atg7*$^{M\Delta}$ mice at any of the doses or time points tested (Figure S4A). To test whether macrophage-mediated antigen presentation was defective in *Atg7*$^{M\Delta}$ mice, we used DOBW cells, which recognize ovalbumin$_{323–339}$ peptide in the context of MHC-II and produce IL-2 upon proliferation (Harding et al., 1991). We could not detect any significant differences in antigen presentation between resident peritoneal macrophages from control and *Atg7*$^{M\Delta}$ mice (Figure S4B).

We also ruled out any gross defects in T cell compartments in *Atg7*$^{M\Delta}$ mice that might account for the phenotype observed (Figures S4C–S4J). Moreover, we found that use of neutrophil-depleting 1A8 antibody, but not isotype control antibody, after the onset of EAE abolished the difference between *Atg7*$^{f/f}$ and *Atg7*$^{M\Delta}$ mice in disease severity, cumulative disease score, and weight loss (Figure S5). Taken together, we concluded that the protective effect in the *Atg7*$^{M\Delta}$ mice was caused by defective neutrophil function and not by changes in peripheral T cell activation.

Autophagy Deficiency Led to Reduced NADPH Oxidase-Mediated ROS Production

Flavocytochrome b_{558}, a component of the NADPH oxidase machinery, is present on the membrane of secondary granules of neutrophils (Jesaitis et al., 1990). Therefore, NADPH oxidase-mediated ROS production in neutrophils depends on fusion of granules with plasma membrane/phagosome. Our in vivo data suggested a fusion defect between granules and phagosome as a cause of reduced degranulation. To further examine how autophagy affected ROS generation by NADPH oxidase, we stimulated control or *Atg7*$^{M\Delta}$ BMN with fMLF and measured extracellular ROS generation by cytochrome *c* reduction assay. *Atg7*$^{M\Delta}$ neutrophils showed significant reduction in ROS production in response to fMLF (Figure 5A). Similarly, FcOxyburst assay also revealed reduced phagosomal ROS production by *Atg7*$^{M\Delta}$ neutrophils (Figure 5B). Similar results were found in *Atg5*$^{M\Delta}$ BMN, in which both phagosomal ROS generation and extracellular ROS generation, measured by OxyBURST Green H_2 HFF BSA, were substantially reduced (Figures 5C and 5D). We did not find any gross difference with respect to fMLF-induced F-actin organization between control and *Atg7*$^{M\Delta}$ neutrophils (data not shown). Collectively, our data suggested that autophagy deficiency reduced NADPH oxidase-mediated ROS production by neutrophils.

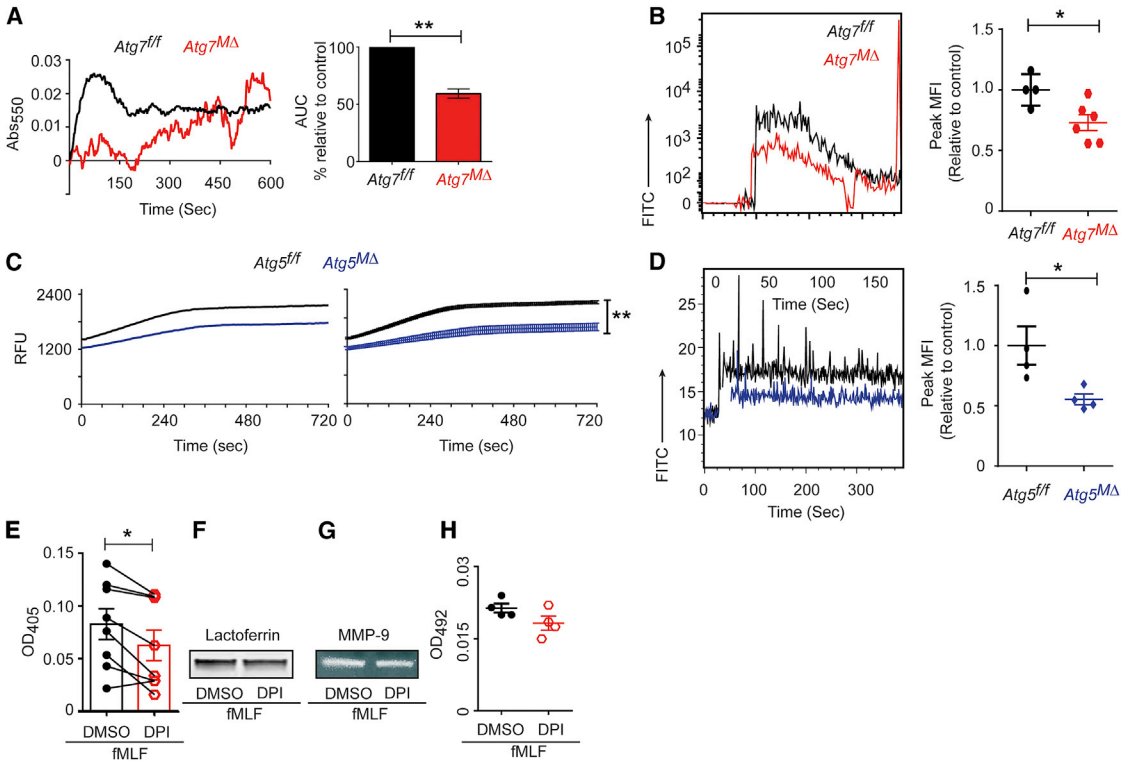

Figure 5. Autophagy Deficiency Reduced NADPH Oxidase-Mediated ROS Production in BMN

(A) BMN from control or *Atg7*$^{M\Delta}$ mice were incubated with cytochrome *c* and extracellular ROS generation in response to 1 μM fMLF was monitored continuously by measuring changes in absorbance (OD_{550}). Data are from four independent experiments.

(B) BMN from control or *Atg7*$^{M\Delta}$ mice were incubated with 125 μg/ml FcOxyburst reagent, and phagosomal ROS generation was measured by flow cytometry. A representative plot and quantitative analyses are shown. Each symbol represents one mouse. Data are from three independent experiments.

(C) BMN from control or *Atg5*$^{M\Delta}$ mice were incubated with OxyBURST H_2HFF Green BSA, and extracellular ROS generation in response to 1 μM fMLF was monitored by measuring changes in fluorescence. Time point 0 is the first time point measured after addition of fMLF. One representative curve for each genotype and quantitative analyses are shown. n = 4/genotype.

(D) BMN from control or *Atg5*$^{M\Delta}$ mice were incubated with 125 μg/ml FcOxyburst reagent, and phagosomal ROS generation was measured by flow cytometry. A representative plot and quantitative analyses are shown. Each symbol represents one mouse. n = 4/genotype.

(E–H) BMN from wild-type mice were pre-treated with DPI (10 μM, 1 hr) and then stimulated with cytochalasin B and fMLF. Supernatant was collected and β-glucuronidase was measured; n = 8/group. Each pair of symbols represents one mouse (E). Supernatant was also subjected to immunoblotting using lactoferrin antibody (F) or gelatinase zymography (G). Representative immunoblots and zymogram are shown. (H) Supernatant was analyzed colorimetrically for LDH activity to detect cell death.

Data are representative of three independent experiments and presented as mean ± SEM; *p < 0.05, **p < 0.01. AUC, area under the curve.

We then investigated the effects of NADPH oxidase-mediated ROS generation on neutrophil degranulation. We pre-treated BMN from wild-type mice with vehicle only or with diphenyleneiodonium chloride (DPI, 10 μM, 1 hr), a specific inhibitor of NADPH oxidase, and then stimulated these neutrophils with cytochalasin B and fMLF. Treatment with DPI reduced degranulation of primary, secondary, and tertiary granules and did not alter cell viability (Figures 5E–5H). Taken together, these data showed that autophagy deficiency led to reduced NADPH oxidase-mediated ROS production and reduced ROS production by NADPH oxidase further contributed to reduced degranulation from *Atg7*$^{M\Delta}$ neutrophils.

DISCUSSION

Despite extensive characterization of neutrophil phagocytic activity and granules, the underlying mechanisms governing functions of neutrophils remain incompletely understood. In this regard, the current study provided several important findings and established autophagy as a regulator of neutrophil functions. It showed that inhibition of autophagy reduced degranulation from neutrophils. Such reduction was not due to decreased production of proteins but rather to a secretion defect. Moreover, deficiency of either *Atg5* or *Atg7* led to similar defects in degranulation from neutrophils, suggesting a role of autophagy, rather than autophagy-independent ATG functions, in mediating degranulation from neutrophils. The in vivo relevance of these findings was confirmed using several mouse models of neutrophil-mediated inflammatory and autoimmune processes, which showed that autophagy deficiency reduced the inflammatory potential of neutrophils.

Autophagy deficiency produced maximal effects on degranulation of tertiary and secondary granules. In contrast, we did not find any defect in upregulation of CD11b, suggesting

intact degranulation of secretory vesicles in absence of autophagy. In neutrophils, secretory vesicles degranulate immediately following mild stimulation. Tertiary and secondary granules follow secretory vesicles, in that order, and primary granules are the most difficult to mobilize. It is possible that the differential effect of autophagy deficiency on different granules is a direct consequence of ease of mobilizing these granules.

We predominantly used fMLF, which is among the strongest inducers of neutrophil degranulation. It is possible that fMLF induction masked any potentially small defect in degranulation of secretory vesicles. However, defective degranulation is unlikely to be specific for fMLF, as both LPS and zymosan resulted in reduced MMP-9 release by autophagy-deficient neutrophils. Thus, our data suggested an intrinsic problem in the degranulation mechanism in absence of autophagy.

Another important finding in this study is the role of NADPH oxidase and ROS in mediating effects of autophagy on degranulation. Our data are consistent with a recent report, which showed similar effects of autophagy in mucus secretion by goblet cells (Patel et al., 2013). However, the potential roles of NADPH oxidase and ROS in mediating neutrophil degranulation remain incompletely elucidated. Studies by different groups using neutrophils isolated from patients of chronic granulomatous disease, in which NADPH oxidase-mediated ROS generation is impaired, provided contrasting observations showing impaired, augmented, or unaltered degranulation (Baehner et al., 1969; Gold et al., 1974; Pak et al., 2007). Such a wide spectrum of results could be attributed to demography of patients studied, types of stimuli used, methods employed, and granular contents measured as an index of degranulation. Although, in our study, inhibition of NADPH oxidase resulted in significant reduction of degranulation, the effects were modest compared to that observed in autophagy-deficient neutrophils, suggesting that additional factors contributed to the effect of autophagy on degranulation.

Assembly of NADPH oxidase requires fusion of secondary granules, the membrane of which contains b_{558} subunit, with phagosomal or plasma membrane (Jesaitis et al., 1990). Autophagy proteins are important for fusion of enzyme-containing lysosomes with plasma membrane in osteoclasts (DeSelm et al., 2011). The role of autophagy in mediating granule fusion is also observed in our study, using in vivo model of PMA-induced ear inflammation. In this model, bioluminescence imaging of neutrophilic MPO by luminol depends on proper fusion of MPO-containing granules with phagosome (Gross et al., 2009). We found substantial reduction in MPO bioluminescence from the PMA-treated ear of *Atg7*$^{M\Delta}$ mice, suggesting a potential defect in fusion of these granules with phagosome. Thus, our study supports a model in which autophagy mediates both fusion of granules with other membrane compartments and degranulation. Autophagy-mediated granule fusion helps in the assembly of an active NADPH oxidase complex and NADPH oxidase-mediated ROS generation, in turn, further facilitates degranulation.

Despite normal transwell migration in response to different stimuli, *Atg7*$^{M\Delta}$ mice showed increased neutrophils in the peritoneal cavity following thioglycollate administration. This finding was likely the result of a higher number of circulating and bone-marrow neutrophils in *Atg7*$^{M\Delta}$ mice. We have previously shown increased proliferative activity of leukocytes in bone marrow of *Atg7*$^{M\Delta}$ mice (Abdel Fattah et al., 2015). Moreover, ROS are critical for several neutrophil death mechanisms and reduced ROS generation in *Atg7*$^{M\Delta}$ neutrophils may also contribute to better survival (Geering and Simon, 2011). Together, these factors could explain higher numbers of circulating neutrophils in *Atg7*$^{M\Delta}$ mice.

Emerging evidence suggests that neutrophils play a broad role in the immune system (Kumar and Sharma, 2010). Neutrophils have been implicated in autoimmunity and as one of the major effector cells in EAE (Rumble et al., 2015). Autophagy has also been implicated in several autoimmune diseases (Bhattacharya and Eissa, 2013). In this regard, we found that autophagy deficiency in myeloid cells reduced severity of EAE. Reduced severity of EAE in myeloid-specific autophagy deficient mice was not due to impairment of peripheral antigen-specific T cell activation. These results are consistent with our recent findings supporting dendritic cells (DCs) as a major antigen presenting cells during EAE (Bhattacharya et al., 2014a). LysMCre-mediated deletion predominantly affects macrophages and neutrophils, whereas DCs remain largely unaffected (Clausen et al., 1999). Normal peripheral T cell activation may reflect unaltered functions of DCs in these mice. Autophagy-deficient neutrophils showed reduced secretion of MMP-9, a matrix metalloproteinase involved in breakdown of blood-CNS barrier in EAE. MMP-9 is considered a potential therapeutic target in multiple sclerosis and clinical trials with MMP-9 inhibitors are currently under way (Muroski et al., 2008). Reduced severity of EAE in *Atg7*$^{M\Delta}$ mice correlated with reduced permeability of blood-spinal cord barrier and reduced CNS cellular infiltration. Although our studies utilized well-characterized neutrophil-mediated in vivo models, they do not exclude possible roles of other myeloid cells in these models.

In summary, this study revealed an important role of autophagy in neutrophils during inflammatory and autoimmune diseases. Neutrophils constitute the major portion of circulating leukocytes in humans and are considered the first line of cellular defense and major inflammatory mediator in the immune system. Neutrophilic secretion is required not only during infection but also to maintain normal homeostasis. Increased neutrophilic secretion has been implicated in the pathogenesis of diseases such as chronic obstructive lung disease and cystic fibrosis. Recent studies proposed alteration of the autophagy pathway as a therapeutic approach to multiple diseases. Our study uncovered autophagy as a major regulator of neutrophil function and suggested the possibility that targeting the autophagy pathway might offer an important approach to treat neutrophil-mediated inflammatory processes.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and LysM-Cre transgenic mice in a C57BL/6 background were purchased from Jackson Laboratory. *Atg7*$^{M\Delta}$ and *Atg5*$^{M\Delta}$ mice have been previously described (Bonilla et al., 2013; Shin et al., 2013; Abdel Fattah et al., 2015). All mice used were housed in BCM vivarium (biosafety level 2). All animal protocols were approved by institutional boards of Baylor College of Medicine.

In Vivo Disease Models

EAE and contact CHS models were induced as described previously (Bhattacharya et al., 2014a). For EAE, active immunization was performed with $MOG_{35–55}$ peptide emulsified in Freund's complete adjuvant, along with two doses of pertussis toxin. Mice were monitored daily for weight changes and clinical signs (Supplemental Experimental Procedures). CHS was induced by sensitizing and challenging the mice with Oxazolone or DNFB. Ear thickness was measured before and 24 hr after the challenge. Acute dermatitis was induced by applying 20 μg of PMA in 20 μl of DMSO to one ear and DMSO only (vehicle) to the other ear, and ear swelling was measured after 24 hr. Bioluminescence imaging was performed as previously described (Gross et al., 2009). In brief, mice were anaesthetized by isoflurane and imaging was performed in a Xenogen In Vivo Imaging System (IVIS), 10 min after intraperitoneal injection of luminol. Quantitation was done by software (Living Image In Vivo Imaging Software, PerkinElmer).

Isolation of Neutrophils from Bone Marrow or Peritoneal Cavity

Single-cell suspension from bone marrow, after ACK lysis, was subjected to gradient centrifugation in 60%/80% Percoll, and neutrophils were collected from the interface (Halpert et al., 2011), washed in HBSS without Ca^{2+} and Mg^{2+}, and stained for flow cytometry or used for in vitro stimulation. Peritoneal lavage was performed with 10 ml of ice-cold HBSS without Ca^{2+} and Mg^{2+} 4 hr after injection of 1 ml thioglycollate, and neutrophils were isolated by Percoll gradient.

BMN Stimulation and Assessing Degranulation

Isolated BMN were allowed to settle for 20–30 min at 37°C, 5% CO_2 and then pre-treated for 5 min with 10 μg/ml of cytochalasin B. BMN were then stimulated with 1 μM fMLF, 1 μg/ml of LPS or PMA, or 10 ng/ml of GM-CSF for various time points. In some experiments, neutrophils were pre-treated for 2 hr with 50 μM CQ and then cytochalasin B was added for 5 min, followed by fMLF stimulation. Supernatant was collected by centrifugation and cells were prepared for flow cytometry. MMP-9 zymography was performed using Novex 10% Zymogram (Gelatin) Gel. For MPO detection, supernatant was incubated with TMB reaction mixture (1 mg of 3,3′,5,5′-tetramethylbenzidine in 1 ml of DMSO and 9 ml of 0.1 M NaH_2PO_4 (pH 5.5) with 2 μl of fresh 30% hydrogen peroxide) for 15 min (in dark) and OD_{615} was measured immediately. For detection of β-glucuronidase, supernatant was incubated overnight with 3.15 mg/ml p-nitrophenyl-itroglucuronide in 0.17 M sodium acetate buffer (pH 4.0), reaction was terminated by adding 0.4 M glycine (pH 10), and OD_{405} was measured immediately.

Measurement of ROS Production

Cytochrome *c* reduction assay and OxyBURST Green H_2HFF BSA assay were performed to detect extracellular ROS production using 0.5×10^6 to 1×10^6 cells/well or 2×10^6 cells/well, respectively in a 96-well plate. Cytochrome *c* or OxyBURST Green H_2HFF BSA was added (final concentration of 1.5 mg/ml or 10 μg/ml, respectively) to the cells and baseline absorbance/fluorescence was recorded. fMLF was then added (final concentration: 1 μM in 200 μl/well) and absorbance/fluorescence was monitored continuously at 550 nm (absorbance) or at 488 nm excitation and 530 nm emission (fluorescence). FcOxyburst assays were performed according to the manufacturer's instructions (Invitrogen). In brief, flow cytometric measurement was performed using a total of 2×10^6 cells/tube. After baseline reading, FcOxyburst reagent was added (125 μg/ml in a final volume of 400 μl), and data were collected continuously with LSR Fortessa (Becton Dickinson). FACSDiva software was used for data collection, and FlowJo software was used for analysis. Cell viability assays were performed using lactate dehydrogenase (LDH) detection kit (Roche).

Statistical Analysis

Normally distributed data were analyzed by Student's t test (Welch correction was applied in case of significant difference in variance between the groups), and non-parametric comparisons were performed by Mann-Whitney test. For EAE data, curves were compared by ANOVA, daily clinical scores and weight changes by Student's t test. Paired t test was used for comparisons between matched pairs (in vivo bioluminescence and degranulation of primary granules after DPI treatment). Analyses were performed by GraphPad Prism 6.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.019.

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