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Toll-like receptors control autophagy

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Autophagy is a newly recognized innate defense mechanism, acting as a cell-autonomous system for elimination of intracellular pathogens. The signals and signalling pathways inducing autophagy in response to pathogen invasion are presently not known. Here we show that autophagy is controlled by recognizing conserved pathogen-associated molecular patterns (PAMPs). We screened a PAMP library for effects on autophagy in RAW 264.7 macrophages and found that several prototype Toll-like receptor (TLR) ligands induced autophagy. Single-stranded RNA and TLR7 generated the most potent effects. Induction of autophagy via TLR7 depended on MyD88 expression. Stimulation of autophagy with TLR7 ligands was functional in eliminating intracellular microbes, even when the target pathogen was normally not associated with TLR7 signalling. These findings link two innate immunity defense systems, TLR signalling and autophagy, provide a potential molecular mechanism for induction of autophagy in response to pathogen invasion, and show that the newly recognized ability of TLR ligands to stimulate autophagy can be used to treat intracellular pathogens.

Introduction

Autophagy is a fundamental cellular homeostatic process, where cells ingest and digest portions of their own cytoplasm, thus periodically cleansing their interiors (Levine and Klionsky, 2004). Autophagy is based on formation within the cytosol of double-membrane organelles termed autophagosomes to sequester portions of the cytoplasm earmarked for autophagic removal or turnover (Mizushima *et al*, 2002). Autophagosomes fuse with lysosomes to form autolysosomes, resulting in degradation of the captured cytosolic constituents, including (i) long-lived proteins and other stable macromolecules and (ii) membranous structures

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such as damaged, spent or surplus organelles (Levine and Klionsky, 2004). Autophagy endows cells with a capability to adjust down their biomass and turn over their own constituents at times of starvation (Lum *et al*, 2005). This provides amino acids and other components for the synthesis of essential proteins and other macromolecules allowing the cells to survive. Autophagy also removes faulty organelles such as spuriously damaged or leaky mitochondria lest cells undergo unscheduled apoptosis and die. All eukaryotic cells from yeast to man are capable of undergoing autophagy, and most cells in the human body can activate this process (Levine and Klionsky, 2004) or even undergo a considerable level of constitutive autophagy (Schmid *et al*, 2007).

Since autophagy affects many cell types, it has a broad effect on a wide range of normal physiological processes, including ageing and diseases such as cancer (Levine, 2007) and neurodegeneration (Alzheimer's, Huntington's and Parkinson's diseases and ataxias; Rubinsztein, 2006). It has recently been recognized that akin to the role of autophagy in eliminating toxic protein aggregates and thus protecting against neurodegeneration (Nixon, 2006), autophagy also plays a role in innate immunity against intracellular pathogens (Deretic, 2005; Levine and Deretic, 2007; Schmid and Munz, 2007), by clearing microbes directly via ingestion into autophagosomes for subsequent degradation in autolysosomes (Gutierrez *et al*, 2004; Nakagawa *et al*, 2004; Ogawa *et al*, 2005; Andrade *et al*, 2006; Orvedahl *et al*, 2007). Agonist-induced autophagosomes capture and destroy intracellular pathogens even when they are safely ensconced in protective vacuoles, such as the immature phagosome harbouring *Mycobacterium tuberculosis* (Singh *et al*, 2006), or parasitophorous vacuoles containing *Toxoplasma gondii* (Andrade *et al*, 2006; Ling *et al*, 2006). In addition to these cell-autonomous protective functions against invading pathogens, autophagy participates in other aspects of immunity (Deretic *et al*, 2006; Levine and Deretic, 2007; Schmid and Munz, 2007) and is not limited to a role in direct elimination of invading bacteria, protozoans and viruses. For example, autophagy supports sequestration of endogenously synthesized viral or self-antigens into autophagosomes and their delivery to major histocompatibility complex (MHC) class II loading compartments, leading to MHC class II-restricted presentation of cytoplasmic antigens (Schmid and Munz, 2007). Autophagy has a role in T-cell homeostasis (Li *et al*, 2006), for example by controlling T-cell lifespan once they exit the thymus (Pua *et al*, 2007), and is an effector of Th1–Th2 polarization in defense against intracellular pathogens (Harris *et al*, 2007). The role of autophagy in immunity has been further underscored by the recent recognition of a genetic association between a Chron's disease-susceptibility locus with one of the core autophagy genes, Atg16 (Hampe *et al*, 2007), and with the human immunity-related GTPase IRGM (Parkes *et al*, 2007) implicated in autophagy (Singh *et al*, 2006).

Two of the remaining top-tier questions in the field of autophagy are how the presence of potential autophagic

targets is detected by the cell and how these targets become earmarked for autophagic degradation. These issue are complicated by the inherent diversity of autophagic targets, represented by a variety of macromolecules, protein aggregates and an assortment of organelles, including mitochondria (Lemasters, 2005), peroxisomes (Farre and Subramani, 2004) and endoplasmic reticulum (Bernales *et al*, 2006). In yeast, unique proteins select the targets for autophagy-related processes known as the Cvt pathway (a specialized zymogen transport and maturation pathway; Kim *et al*, 2001) and pexophagy (autophagy of peroxisomes; Rayapuram and Subramani, 2006). In mammalian cells, the initial information points towards ubiquitylation, suggesting that ubiquitin may be a part of the molecular recognition at least in the case of protein aggregates (Bjorkoy *et al*, 2005). While this issue has only begun to be addressed for endogenous cellular targets, even less is known regarding signals that lead to the recognition of intracellular pathogens. In this work, we have considered the possibility that innate immunity pattern recognition receptors (PRRs) have a role in activation of autophagy upon detection of pathogen molecules. PRRs such as Toll-like receptors (TLRs), Nods and Nod-like receptors (NLRs), RIG-I and RIG-I-like receptors (RLRs) detect the presence of microbial invaders by recognizing pathogen-associated molecular patterns (PAMPs; Medzhitov, 2007). PRRs specialize for a subset of cellular compartments that they patrol and for unique classes of microbial products that they recognize. It is generally believed that topologically external spaces, including vacuolar compartments, are covered by one or a combination of different TLRs, whereas the cytosol is under the surveillance by NLR and RLR molecular complexes. It has been shown that autophagic machinery can deliver PAMPs to endosomal TLRs (Li *et al*, 2006), but the question of whether the reverse is true, that is, whether PAMPs can stimulate autophagy once recognized by TLR molecules, has not been addressed. Here we report a screen with a panel of PAMPs for their capacity to induce autophagy and show that a subset of TLR ligands can activate autophagy. The TLR7 ligand single-stranded RNA (ssRNA) and other TLR7 agonists are potent inducers of autophagy. This validates the hypothesis that PRR-based recognition of PAMPs serves as a detector for microbial presence and induces autophagy as a defense mechanism against invading pathogens, thus bridging the two seemingly separate innate-immunity systems.

Results

TLR ligands induce formation of LC3 puncta in macrophages

Autophagy induction in various systems is invariably monitored by an assay that depends on translocation of the autophagosome protein LC3 (Atg8) from the cytosol (diffuse cytosolic distribution) to newly formed autophagosomes, which appear as cytoplasmic puncta (Kabeya *et al*, 2000). To test whether recognition of microbial PAMPs via TLR molecules can induce an autophagic response, we screened a panel of standard PAMP ligands at standard concentrations (see Supplementary data) in macrophages for induction of LC3 puncta. RAW 264.7 macrophages were transfected with green fluorescent protein–LC3 (GFP–LC3) and 24 h later incubated in starvation media (positive control for autophagy

induction), or in complete media with or without individual PAMPs. As a measure of autophagy, GFP–LC3 puncta ($\geqslant 1\,\mu m$) were quantified in >100 cells per sample (Figure 1; Supplementary Figure S1), in the absence of any further manipulation (Figure 1A–C) or in the presence of autolysosomal protease inhibitors, to prevent degradation of formed GFP–LC3 puncta (Supplementary Figure S1). A significant induction of GFP–LC3 puncta formation was observed with TLR3, TLR4 and TLR7 ligands. No significant increase in LC3 puncta was detected with synthetic bacterial lipopeptides, Pam_3CSK_4 (TLR1/TLR2 ligand) and Pam_2CSK_4 (TLR2/TLR6 ligand), *Salmonella typhimurium* flagellin (TLR5 ligand) and a CpG oligonucleotide (TLR9 ligand; Figure 1A and B; Supplementary Figure S1). Poly(I:C), a TLR3 ligand, and lipopolysaccharide (LPS), a TLR4 ligand, evoked an increase in GFP–LC3 puncta (Figure 1A and B). To ascertain TLR's presence and their responsiveness to all ligands tested, and thus validate those results that were negative, we carried out an nuclear factor-κB (NF-κB) activation assay using a NF-κB–luciferase reporter construct. All TLR ligands tested, with the exception of poly(I:C) and bacterial flagellin, induced NF-κB activation (Supplementary Figure S2A). Poly(I:C) was nevertheless able to stimulate the cells, as detected by induction of interferon-β (IFN-β) secretion (Supplementary Figure S2B), although no IκB-α degradation or Janus kinase (JNK) phosphorylation were detected (Supplementary Figure S2D and E). Whereas TLR5 (flagellin receptor) was present in RAW 264.7 macrophages, as shown by western blotting (Supplementary Figure S2C), no NF-κB activation (Supplementary Figure S2A), IκB-α degradation or JNK phosphorylation was detected with flagellin (Supplementary Figure S2D and E). Activation of NF-κB, detected by direct measurement (Supplementary Figure S2A) or in correlation with IκB-α degradation (Supplementary Figure S2D), or transient JNK phosphorylation (Supplementary Figure S2D and E), detected for all other TLR ligands, was however not sufficient to induce LC3 puncta formation (Figure 1A and B; Supplementary Figure S1). Although no induction of GFP–LC3 puncta was observed after TLR2 activation with individual lipopeptides (Figure 1B; Supplementary Figure S3A), zymosan (a more complex TLR2 agonist engaging additional receptors) was a strong inducer of GFP–LC3 puncta (Supplementary Figure S3A and B), indicating that in some instances a combination of receptors was required for induction of autophagy.

ssRNA, a TLR7 ligand, induced the most prominent increase in LC3 puncta formation (Figure 1A and B). Stimulation with ssRNA was comparable to starvation used as a gold standard for autophagy induction (Seglen and Bohley, 1992), or to the previously reported (Gutierrez *et al*, 2004) induction with an immunologically relevant agonist IFN-γ (Figure 1A and B). We further examined whether stimulation of TLR7 induced autophagy, by employing a different TLR7 ligand, imiquimod (R837), an imidazo-quinoline amine guanosine analogue often used as a conventional TLR7 ligand (Hemmi *et al*, 2002; Lee *et al*, 2003). Increased formation of LC3 puncta was detected upon stimulation with imiquimod (Figure 1A and C). This was additionally confirmed by four-dimensional live confocal microscopy (Supplementary Movie 1).

To confirm that GFP–LC3 puncta formation induced by ssRNA and imiquimod is a reflection of autophagy activation
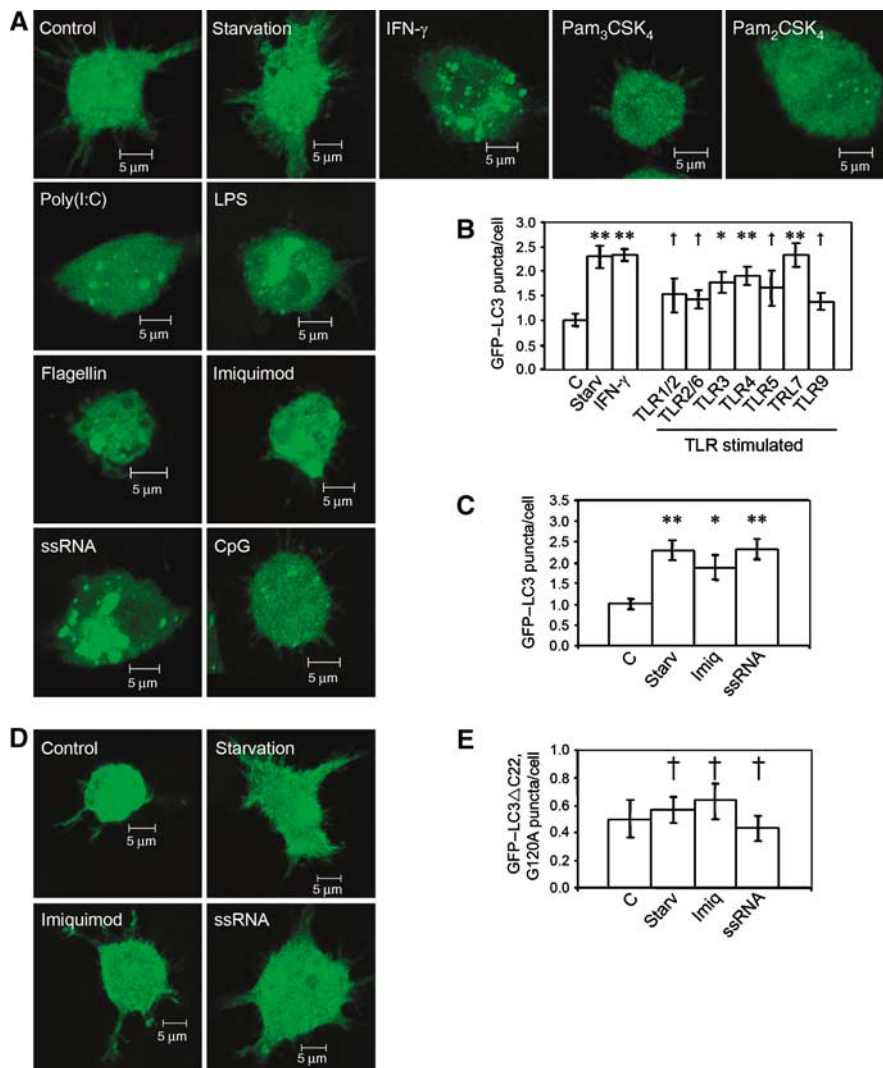
Figure 1 TLR7 ligands are strong inducers of LC3 puncta. (**A**) RAW 264.7 macrophages cells were transfected with GFP–LC3 and after 24 h cells were incubated for 2 h in starvation media or for 4 h in complete media alone or in the presence of 500 U/ml IFN-γ, 1 µg/ml Pam$_3$CSK$_4$, 100 ng/ml Pam$_2$CSK$_4$, 25 µg/ml poly(I:C), 500 ng/ml LPS, 1 µg/ml *S. typhimurium* flagellin, 10 µg/ml Imiquimod, 10 µg/ml ssRNA or 3 µM CpG oligonucleotide 1826. Bar, 5 µm. (**B**, **C**) Quantification of GFP–LC3 puncta ($\geqslant 1$ µm) in RAW 264.7 macrophages in panel A. Data are means $\pm$ s.e.m. ($n \geqslant 3$); **$P < 0.01$, *$P < 0.05$, $^{\dagger}P \geqslant 0.05$ (analysis of variance (ANOVA)). (**D**) RAW 264.7 macrophages cells were transfected with GFP–LC3ΔC22,G120A and after 24 h cells were incubated for 2 h in starvation media (Starv) or 4 h in complete media alone (C) or in the presence of 10 µg/ml imiquimod (Imiq) or 10 µg/ml ssRNA. Bar, 5 µm. (**E**) Quantification of GFP–LC3ΔC22,G120A puncta ($\geqslant 1$ µm) in RAW 264.7 macrophages in panel D. Data are means $\pm$ s.e.m. ($n = 6$); $^{\dagger}P \geqslant 0.05$ (ANOVA).

and not nonspecific protein aggregation (Kuma *et al*, 2007), we employed a control based on GFP fusion with a mutant LC3 form, LC3ΔC22,G120A (Kabeya *et al*, 2000). This LC3 mutant lacks the last 22 residues and a C-terminal glycine, disabling it for C-terminal lipidation with phosphatidylethanolamine (PE) by the autophagic Atg7–Atg3–PE conjugation system (Kabeya *et al*, 2000). The PE posttranslational modification of the C-terminal Gly residue in LC3 is required for LC3 association with autophagic membranes and autophagosome elongation (Kabeya *et al*, 2000). Figure 1D and E show that the number of GFP–LC3Δ22,G120A puncta per cell did not change upon stimulation with imiquimod, ssRNA or starvation, demonstrating that GFP–LC3 puncta formation following addition of TLR7 ligands in our experiments was dependent on a functional LC3 association with growing autophagosomes.

TLR7 ligands induce autophagy

To demonstrate that the process detected as an increase in LC3 puncta was indeed a bona fide autophagy, we carried out a panel of additional experiments and assays. Beclin 1 (Liang *et al*, 1999) is a critical autophagic protein (Atg6) required for induction of autophagy and execution of the various stages along the autophagic pathway. The TLR7 ligands imiquimod and ssRNA, as well as the starvation control, induced fewer GFP–LC3 puncta (Figure 2A) when Beclin 1 levels were knocked down by short interfering RNA (siRNA) (Figure 2B), Thus, imiquimod and ssRNA induced autophagy in a Beclin 1-dependent manner.

We next examined the autophagic response in primary cells, using murine bone marrow macrophages (BMMs) derived from knock-in transgenic mice expressing GFP–LC3 (Mizushima *et al*, 2004). Unlike the transiently transfected
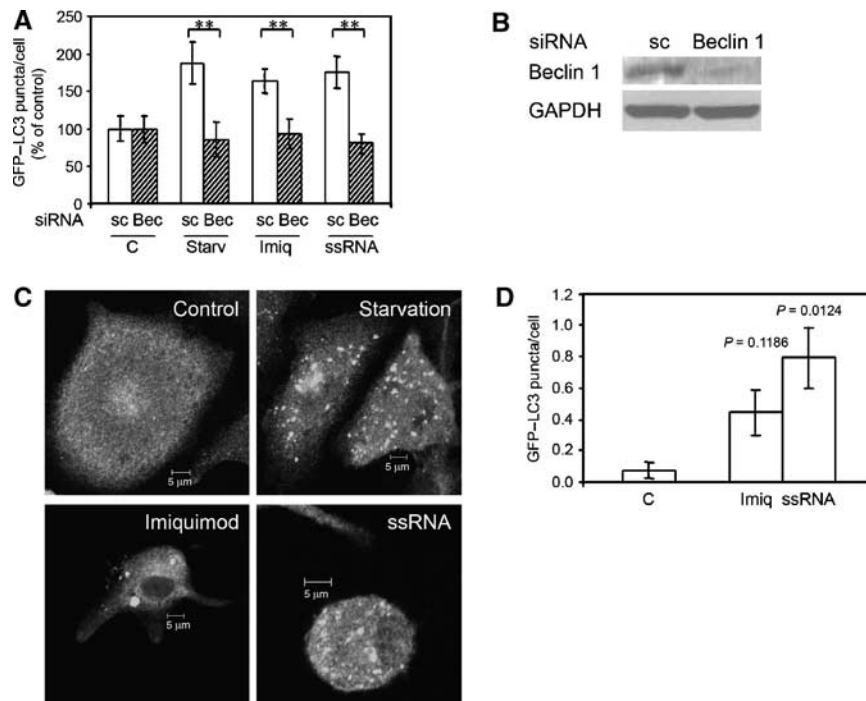
Figure 2 TLR7 ligands induce autophagy. (**A**) Quantification of GFP–LC3 puncta ($\geqslant 1\,\mu m$) in RAW 264.7 macrophages cells co-transfected with GFP–LC3 and either control scrambled siRNA (sc) or Beclin 1 siRNA. After 22 h cells were incubated for 4 h with complete media alone (C) or in the presence of $10\,\mu g/ml$ imiquimod (Imiq) or $10\,\mu g/ml$ ssRNA, or incubated for the last 2 h in starvation medium (Starv). Data are means $\pm$ s.e.m. ($n = 6$); **$P < 0.01$ (ANOVA). (**B**) RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or Beclin 1 siRNA and after 24 h cells were lysed and analysed by western blotting using anti-Beclin 1 or anti-GAPDH antibodies. (**C**) Primary BMMs derived from GFP–LC3 transgenic mice were incubated for 2 h with starvation media or 4 h with complete media alone (control) or in the presence of $10\,\mu g/ml$ imiquimod or $10\,\mu g/ml$ ssRNA. Bar, $5\,\mu m$. (**D**) Quantification of GFP–LC3 puncta ($\geqslant 1\,\mu m$) in BMMs expressing GFP–LC3 in panel C. Cells were stimulated with complete media alone (C), imiquimod (Imiq) or ssRNA. Data are means $\pm$ s.e.m. ($n = 3$). Exact *P*-values (ANOVA) are shown. A full-colour version of this figure is available at The *EMBO Journal* Online.

cells, primary macrophages expressing endogenous GFP–LC3 displayed none or very few background GFP–LC3 puncta when not stimulated for autophagy (Figure 2C and D). When autophagy was stimulated by starvation, BMMs showed increase in GFP–LC3 puncta (Figure 2C). Imiquimod and ssRNA elicited mild GFP–LC3 puncta response (Figure 2C and D). These data indicate that although primary macrophages can react to TLR stimulation by increase in autophagy markers, this response may be under a tighter control in primary cells relative to RAW 264.7 cells.

LC3-I lipidation, which is a hallmark of autophagy induction, can be monitored by electrophoretic mobility shift from the slower migrating, non-lipidated LC3-I form to the lipidated and faster moving LC3-II form (Kabeya *et al*, 2000). We used this autophagy assay to examine TLR ligand effects on murine primary macrophages (BMM) (Figure 3A) and murine macrophage cell lines (RAW 264.7 and J774; Figure 3B and C). Immunoblot analysis of LC3 bands was carried out in the presence of Bafilomycin A$_1$ (Mizushima and Yoshimori, 2007), an inhibitor of autophagic degradation/flux. The TLR ligands Poly(I:C) (TLR3), LPS (TLR4) and ssRNA (TLR7) induced LC3-II conversion, whereas Pam$_3$CSK$_4$ (TLR1/TLR2) and flagellin (TLR5) did not (Figure 3A), in keeping with the results of the LC3 puncta assay (Figure 1). A positive response to the TLR7 ligand ssRNA was also detected in RAW 264.7 (Figure 3B) and J774 (Figure 3C) cells monitored at different times following ssRNA challenge. The response to TLR7 ligands was further confirmed in the absence of inhibitors of autophagic degradation with ssRNA

(Supplementary Figure S4A) and imiquimod (Supplementary Figure S4B). As positive control for autophagy induction, rapamycin (Supplementary Figure S4A and B) and IFN-γ (Supplementary Figure S4B) were used to treat RAW 264.7 cells. The identity of LC3-I and LC3-II bands was confirmed (due to appearance of an additional band in Figure 3B and C) by siRNA knockdown of LC3B (Supplementary Figure S4A, lane 1), one of the five murine Atg8 paralogues (MAP1LC3A, MAP1LC3B, GABARAP, GABARAPL1, GABARAP); LC3B is the mammalian Atg8 invariably monitored in autophagic studies (Kabeya *et al*, 2004). In conclusion, the TLR7 ligands induced conversion of LC3 from LC3-I to LC3-II, consistent with induction of autophagy.

Next, we monitored PAMP-induced autophagy by two LC3-independent criteria, (i) detection of autophagic organelles by ultrastructural analysis using electron microscopy and (ii) autophagic proteolysis of stable polypeptides. An ultrastructural analysis of RAW 264.7 cells treated with ssRNA for 2 or 4 h revealed presence of typical autophagosomal profiles at later stages of maturation, as shown in Figure 4 and in Supplementary Figure S5A and B and enlarged sections, where electron-dense material (denser than the cytoplasm surrounding the vacuole) was seen, characteristically present in autolysosomes due to ribosomal degradation (Eskalinen, 2008). These organelles were absent from the control untreated cells (Figure 4A). A stereometric quantification of organellar surface density in a volume (S_v) (Weibel and Bolender, 1973) showed that, upon stimulation with ssRNA, only autophagic vacuoles increased their S_v value from
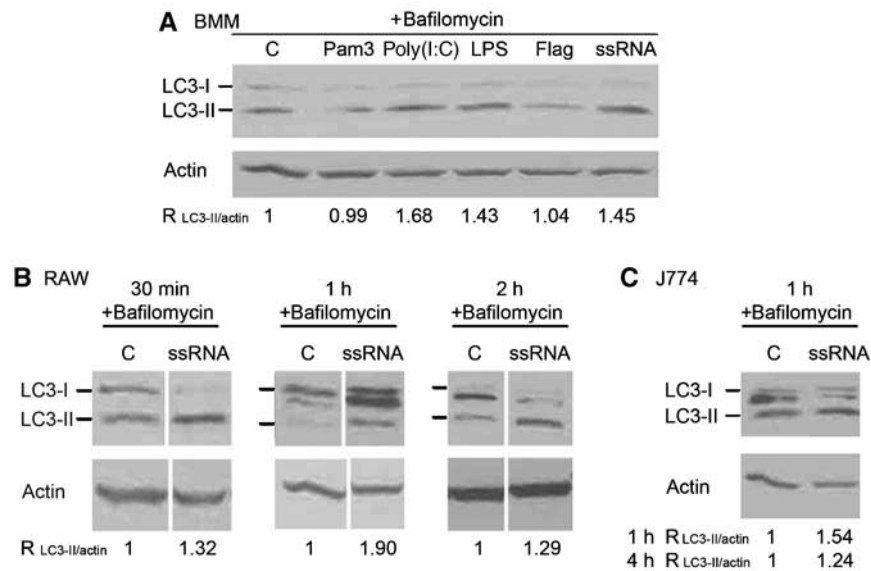
Figure 3 Immunoblot analysis of LC3-I to LC3-II conversion in cells stimulated with TLR ligands. (**A**) Primary BMMs were incubated in the presence of 100 nM Bafilomycin A for 2 h in complete media alone (C) or in the presence of 1 µg/ml Pam$_3$CSK$_4$, 25 µg/ml poly(I:C), 500 ng/ml LPS, 1 µg/ml *S. typhimurium* flagellin or 10 µg/ml ssRNA. Cells were lysed and analysed by immunoblotting using anti-LC3 or anti-actin antibodies. Densitometric LC3-II/actin ratios are shown (average from three experiments). (**B**) RAW 264.7 macrophages were incubated in the presence of 100 nM Bafilomycin A for 30 min, 1 h or 2 h in complete media alone (**C**) or in the presence of 10 µg/ml ssRNA. Cells were lysed and analysed by immunoblotting using anti-LC3 or anti-actin antibodies. Densitometric LC3-II/actin ratios are shown as averages from two (30 min and 2 h) or three independent experiments (1 h). (C) J774 macrophages were incubated in the presence of 100 nM Bafilomycin A for 1 h in complete media alone (C) or in the presence of 10 µg/ml ssRNA. Cells were lysed and analysed by immunoblotting using anti-LC3 or anti-actin antibodies. Blot, 1 h sample. Densitometric LC3-II/actin ratios for 1 and 4 h incubation time points are shown underneath the blot.

0.06 ± 0.03 to $0.34 \pm 0.07 \, \mu^{-1}$, whereas mitochondria or nuclei did not show a statistically significant change in their S_v values (Figure 4C).

Degradation of long-lived proteins represents an end-point autophagy assay, relying on macroautophagic proteolysis of stable polypeptides. It is often used to examine whether a given process represents autophagy and to test whether the autophagic pathway has been executed in full, as it depends on conversion of autophagosomes into degradative autolysosomal organelles (Meley *et al*, 2006). Increased proteolysis of stable polypeptides was observed when autophagy was induced by starvation (Figure 4D). Upon stimulation of cells with the TLR7 ligand imiquimod, a similar increase in degradation of stable proteins was detected (Figure 4D). In control samples, employing autophagic proteolysis inhibitors E-64d and pepstatin A, both starvation- and imiquimod-induced degradation of stable proteins were equally inhibited by this treatment (Figure 4E). A full (with the exception of LPS and zymosan) PAMP panel analysis for induction of stable protein degradation (Supplementary Figure S6) was in keeping with the results obtained with LC3 assays. The results of ultrastructural analyses, proteolysis assays, LC3 puncta and LC3 lipidation tests, and dependence on Beclin 1 demonstrate that TLR7 ligands induce autophagy.

ssRNA induces autophagy through TLR7

After demonstrating that imiquimod and ssRNA induce autophagy, we examined whether these compounds activated autophagy via TLR7. TLR7 was knocked down in RAW 264.7 cells with siRNA (Figure 5A–C). Macrophages were stimulated with a TLR7 ligand and autophagy induction quantified by GFP–LC3 puncta assay (Figure 5A and B). Cells were

incubated in complete media in the absence or presence of ssRNA and compared with starved cells. Although necessary manipulations increased background levels of LC3 puncta in some experiments, there was significant induction of autophagy upon starvation or ssRNA stimulation in cells transfected with the control scrambled siRNA compared with cells transfected with TLR7 siRNA (Figure 5A and B). Importantly, TLR7 knockdown did not change LC3 response to starvation (Figure 5B, inset), demonstrating that TLR7 was required specifically for TLR ligand-induced autophagy, but not for starvation-induced autophagy. As a further control, cells transfected with scrambled siRNA or with TLR7 siRNA had the same level of GFP–LC3 puncta in the complete medium (not shown). TLR7 knockdown, relative to scrambled siRNA control, also diminished LC3-II levels assessed by immunoblotting upon stimulation with ssRNA (Supplementary Figure S7A) or imiquimod (Supplementary Figure S7B). Electron microscopic morphometry of autophagosomal organelles induced by ssRNA indicated that TLR7 knockdown by siRNA diminished S_v from 0.49 ± 0.1 to $0.19 \pm 0.05 \, \mu^{-1}$ (Supplementary Figure S5D). Thus, induction of autophagy by ssRNA and imiquimod is TLR7-dependent.

TLR7 stimulation activates autophagy via MyD88

As all known effector functions of TLR7 signalling depend on the MyD88 adapter protein (Lee and Kim, 2007), we next tested whether MyD88 was required for TLR7 induction of autophagy (Figure 6A–C). Figure 6C shows a temporal analysis of MyD88 knockdown, with optimal MyD88 knockdown being achieved early, at 24 h post siRNA transfection. Macrophages were co-transfected with GFP–LC3 along with control (scrambled) siRNA or MyD88 siRNA.
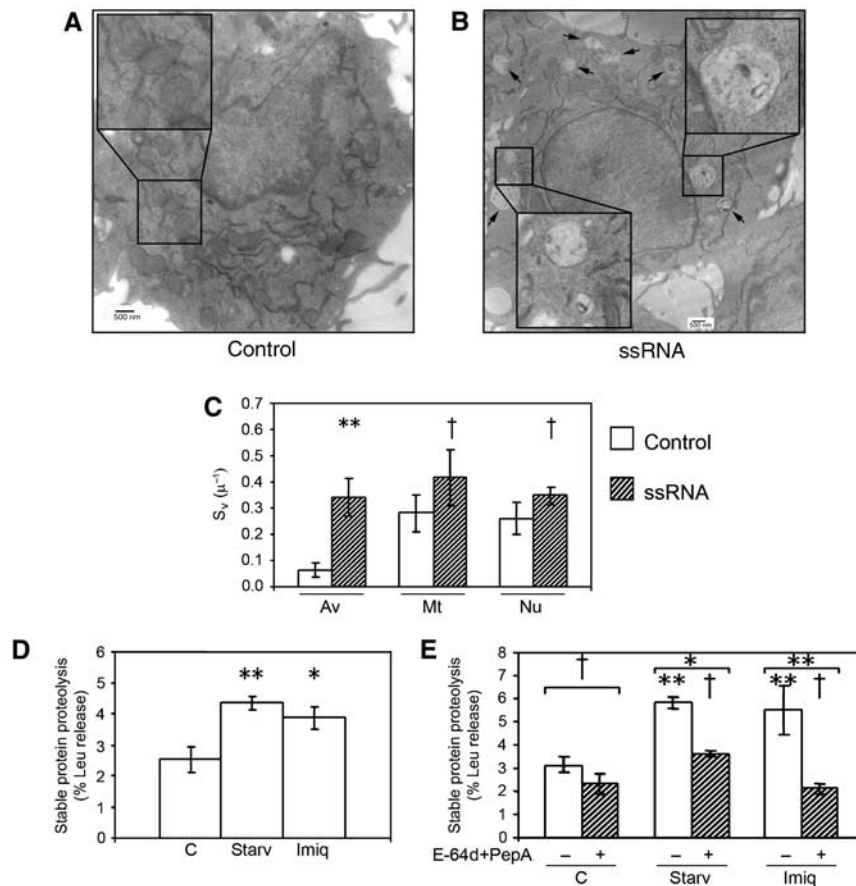
Figure 4 Assessment of autophagy induction with TLR7 ligands by ultrastructural analysis and by monitoring degradation of long-lived proteins. (**A**, **B**) Electron microscopy of RAW 264.7 macrophages incubated for 4 h with (A) complete media alone (control) or (B) in the presence of 10 μg/ml ssRNA. Arrows and enlarged areas indicate autophagic organelles. (**C**) Quantification of organellar surface in a volume (S_v) (Weibel and Bolender, 1973) for autophagic vacuoles (Eskalinen, 2008), mitochondria and nuclei. $**P < 0.05$, $^\dagger P \geqslant 0.05$ (ANOVA). (**D**) Proteolysis of long-lived proteins was measured in RAW 264.7 cells labelled for 24 h in media containing [^{3}H]leucine. Cells were washed, incubated for 24 h in complete medium (containing cold leucine) and incubated in starvation media (Starv) for 4 h or in full media alone (C) or in complete media supplemented with 10 μg/ml imiquimod (Imiq) for 24 h. Leucine release was calculated from radioactivity in the tricarboxylic acid-soluble form relative to total cell radioactivity. Data are means ± s.e.m. ($n = 9$); $**P < 0.01$, $*P < 0.05$ (ANOVA). (**E**) Proteolysis of long-lived proteins was measured in RAW 264.7 cells labelled as in panel D, with 1 h of preincubation with 10 μg/ml E-64d and 10 μg/ml pepstatin A in complete media before stimulation, and stimulated as in panel D but in the presence of 10 μg/ml E-64d and 10 μg/ml pepstatin A. Data are means ± s.e.m. ($n = 3$); $**P < 0.01$, $*P < 0.05$, $^\dagger P \geqslant 0.05$ (ANOVA) relative to the corresponding control. Symbols placed over the lines indicate significance between samples from same condition group with and without E-64d + pepstatin A; symbols under the lines indicate statistical significance relative to control (C).

Following a 24-h incubation period, cells were maintained for 4 h in complete medium (control), or in complete medium supplemented with imiquimod or ssRNA. Another sample treated with siRNA was run in parallel and was subjected to starvation for the last 2 h. Treatment of cells with imiquimod, ssRNA and starvation stimulated LC3 puncta with equal potency in cells co-transfected with the scrambled siRNA (Figure 6A and B). However, in cells subjected to MyD88 knockdown, induction of autophagy was abrogated specifically in response to imiquimod or ssRNA, but not starvation (Figure 6A and B). These relationships were further confirmed by LC3 immunoblotting, using imiquimod and ssRNA in the presence of inhibitors of autolysosomal degradation (Supplementary Figure S8), with MyD88 knockdown diminishing relative amounts of LC3-II under induction conditions. These results demonstrate that induction of autophagy via TLR7 requires MyD88 as a downstream adapter, similar to other, previously known effector functions of TLR7.

Induction of autophagy by TLR7 ligands can eliminate intracellular pathogens

Starvation-induced autophagy can kill intracellular mycobacteria (Gutierrez et al, 2004). As our results presented here indicated that TLR7 was a potent inducer of autophagy, we wondered whether TLR7 ligands could be used as heterologous ligands to induce anti-mycobacterial effects in macrophages, with potential therapeutic implications. We tested whether stimulation of autophagy with TLR7 ligands can reduce viability of mycobacteria in infected macrophages. Following phagocytosis of *M. tuberculosis* var. *bovis* Bacille Calmette-Guérin (BCG), macrophages were stimulated for 4 h with TLR7 ligands or subjected to starvation. After incubation, cells were lysed and lysates plated for colony-forming unit counts. Imiquimod or ssRNA treatment enabled macrophages to decrease BCG survival (Figure 7A). Treatment of infected cells with Pam$_2$CSK$_4$ or CpG (TLR ligands not inducing autophagy in our system) did not affect BCG
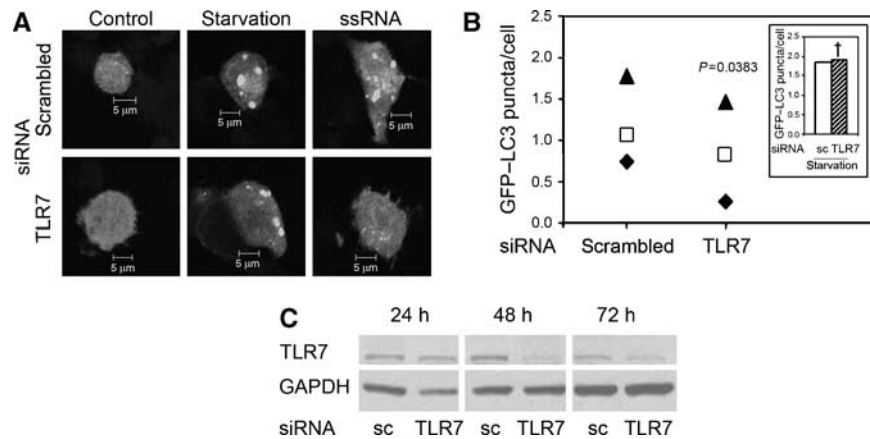
Figure 5 TLR7 is responsible for ssRNA-induced autophagy. (**A**) RAW 264.7 macrophages cells were co-transfected with GFP-LC3 and control scrambled (sc) or TLR7 siRNA. After 46 h, cells were incubated for 4 h with complete media alone (control), in the presence of 10 µg/ml ssRNA (ssRNA), or incubated for the last 2 h in the starvation medium (starvation). Bars, 5 µm. (**B**) Quantification of GFP–LC3 puncta ($\geqslant 1$ µm) in RAW 264.7 macrophages transfected as in panel A and stimulated with 10 µg/ml ssRNA. Inset, quantification of GFP–LC3 puncta in RAW 264.7 macrophages transfected in panel A and stimulated 2 h in starvation media. Symbols denote paired data from same experiments: ▲, experiment 1; □ experiment 2; ◆ experiment 3. *P*-value, paired *t*-test. (**C**) RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or TLR7 siRNA and after 24, 48 or 72 h cells were lysed and analysed by western blotting using anti-TLR7 or anti-GAPDH antibodies. A full-colour version of this figure is available at The *EMBO Journal* Online.
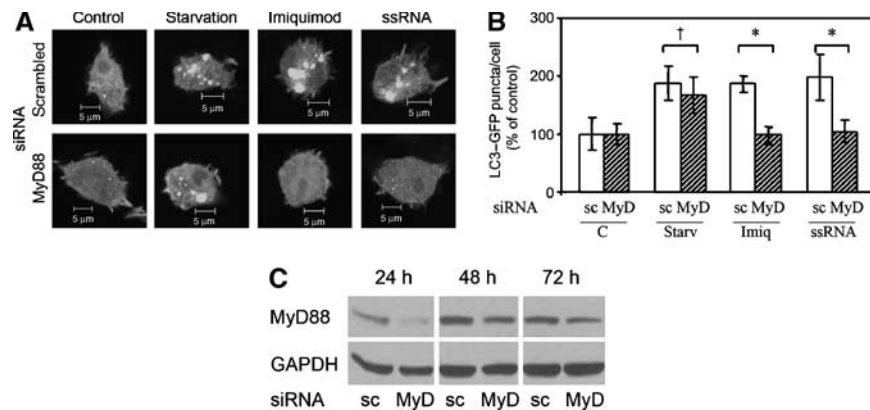

Figure 6 Autophagy induced by TLR7 ligands depends on MyD88. (**A**) Confocal microscopy images of RAW 264.7 macrophages co-transfected with GFP-LC3 and control scrambled siRNA (scrambled) or MyD88 siRNA (MyD88). After 22 h, cells were incubated for 4 h in complete media alone (control) or in complete media supplemented with 10 µg/ml imiquimod or 10 µg/ml ssRNA, or incubated for the last 2 h in the starvation medium. Bars, 5 µm. (**B**) Quantification of GFP–LC3 puncta ($\geqslant 1$ µm) from experiments illustrated in panel A. Data are means $\pm$ s.e.m. ($n = 3$); *$P < 0.05$, †$P \geqslant 0.05$ (ANOVA). (**C**) RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or MyD88 siRNA (MyD) and after 24, 48 or 72 h cells were lysed and analysed by immunoblotting using anti-MyD88 or anti-GAPDH antibodies. A full-colour version of this figure is available at The *EMBO Journal* Online.

survival significantly (Supplementary Figure S9). We next established that stimulation with imiquimod or ssRNA caused BCG killing via TLR7, comparing cells subjected to TLR7 siRNA knockdown with those transfected with scrambled control siRNA (Figure 7B). In cells with TLR7 knockdown, differences in BCG survival following imiquimod or ssRNA stimulation were abrogated (Figure 7B). The effect of TLR7 knockdown did not affect autophagic BCG killing induced by starvation (Figure 7B). Similarly, knockdown of MyD88 also abrogated TLR7-ligand induced BCG elimination, but did not affect starvation-induced autophagic killing of BCG (Figure 7C). Finally, the effects on BCG survival in macrophages stimulated with TLR7 ligands were dependent on autophagic machinery, as Atg5 knockdown

diminished BCG elimination upon ssRNA treatment, showing, as expected, effects similar to those when autophagy was induced by starvation (Figure 7D). A knockdown of another autophagy factor, Beclin 1, completely abrogated the killing effects of starvation and ssRNA treatments (Figure 7F). Thus, a TLR agonist that can induce autophagy in infected macrophages can control an infection caused by a pathogen not normally associated with stimulation of TLR7. This suggests a potential therapeutic use of TLR ligands in treatment of non-cognate infectious agents through induction of autophagy as a generic cell-autonomous mechanism for elimination of intracellular microbes.

We also tested whether infection with a pathogen known to present natural ligands to TLR7 or TLR8 can induce
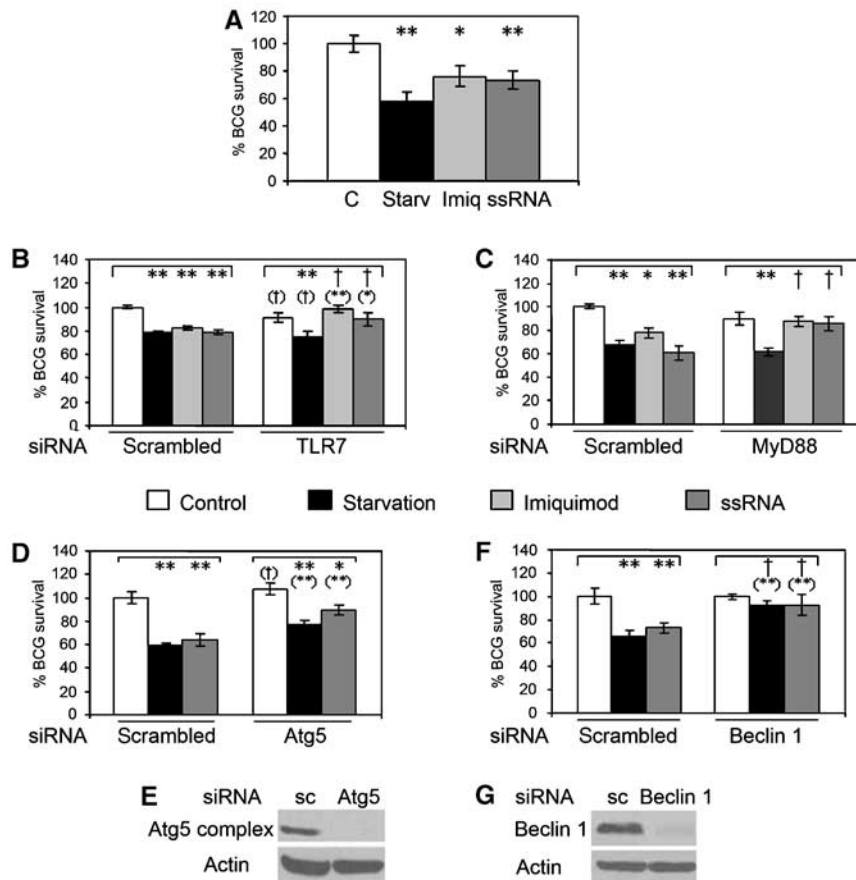
Figure 7 TLR-induced autophagy eliminates intracellular BCG. (**A**) RAW 264.7 macrophages were infected with BCG for 1 h, washed and incubated for 4 h in complete media alone (C) or in the presence of 10 μg/ml imiquimod (Imiq) or 10 μg/ml ssRNA, or incubated in starvation medium (Starv). Cells were lysed to quantify bacterial survival by counting colony-forming units. Data are means ± s.e.m. ($n = 5$); ** $P < 0.01$, * $P < 0.05$ (ANOVA). (**B**) RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or TLR7 siRNA. After 46 h cells were infected, washed, incubated for 4 h and lysed as in panel A. Data are means ± s.e.m. ($n = 3$); ** $P < 0.01$, * $P < 0.05$, † $P \geqslant 0.05$ (ANOVA) relative to control. Symbols in parentheses indicate significance relative to the equally treated cells from the scrambled siRNA group. (**C**) RAW 264.7 macrophages were transfected with control scrambled siRNA (sc) or MyD88 siRNA. After 24 h cells were infected, washed, incubated for 4 h and lysed as in panel A. Data are means ± s.e.m. ($n = 6$); ** $P < 0.01$, * $P < 0.05$, † $P \geqslant 0.05$ (ANOVA) relative to control. (**D**) RAW 264.7 macrophages cells were transfected with control scrambled siRNA (sc) or Atg5 siRNA. After 24 h cells were infected, washed, incubated for 4 h and lysed as in panel A. Data are means ± s.e.m. ($n = 6$); ** $P < 0.01$, * $P < 0.05$, † $P \geqslant 0.05$ (ANOVA) relative to control. Symbols in parentheses indicate statistical significance relative to the equally treated cells from the scrambled siRNA group. (**E**) RAW 264.7 macrophages cells were transfected as in panel D. After 24 h cells were lysed and analysed by immunoblotting using anti-Atg5 or anti-actin antibodies. (**F**) RAW 264.7 macrophage cells were transfected with control scrambled siRNA (sc) or Beclin 1 siRNA. After 24 h, cells were infected, washed, incubated for 4 h and lysed as in panel A. Data are means ± s.e.m. ($n = 6$); ** $P < 0.01$, * $P < 0.05$, † $P \geqslant 0.05$ (ANOVA) relative to control. Symbols in parentheses indicate significance relative to the equally treated cells from the scrambled siRNA group. (**G**) RAW 264.7 macrophages cells were transfected as in panel F. After 24 h cells were lysed and analysed by immunoblotting using anti-Beclin 1 or anti-actin antibodies.

autophagy. One such infectious agent is HIV, with HIV-derived guanosine- and uridine-rich ssRNA known to stimulate TLR7/TLR8 in human cells (Heil *et al*, 2004). The role for TLR7/TLR8 stimulation in HIV infection has been implicated both as an antiviral defense during acute HIV infection (Beignon *et al*, 2005; Schlaepfer *et al*, 2006) and in development of AIDS through immune activation (Meier *et al*, 2007) and replication of the latent virus (Schlaepfer *et al*, 2006). When HeLa cells were infected with vesicular stomatitis virus-G-pseudotyped HIV virus, we detected increase in relative levels of LC3-II (Supplementary Figure S10A and B). The increase in LC3-II levels, indicative of autophagy induction, was TLR8-dependent, as TLR8 knockdown with siRNA abrogated relative increase in LC3-II (Supplementary Figure S10B). Thus, autophagy induction in response to natural TLR7 or TLR8 ligands can be detected during infection with HIV.

Discussion

Autophagy has a role in innate and adaptive immunity (Levine and Deretic, 2007; Schmid and Munz, 2007) as a mechanism for elimination of intracellular bacteria, including *M. tuberculosis*, *Listeria monocytogenes*, *Streptococcus pyogenes*, *Salmonella* and *Shigella* (Rich *et al*, 2003; Gutierrez *et al*, 2004; Nakagawa *et al*, 2004; Ogawa *et al*, 2005; Birmingham *et al*, 2006); viruses such as Herpes simplex virus (Orvedahl *et al*, 2007) and protozoans exemplified by *T. gondii* (Rich *et al*, 2003; Andrade *et al*, 2006; Ling *et al*, 2006). This is in keeping with the cellular maintenance function of autophagy and its role in removal of harmful objects from the cytoplasm (Deretic, 2005). The signals that activate autophagy and molecular tags that guide autophagosomes to sequester the invading pathogens are a matter of current investigations. A large body of literature points to the

capability of cells to recognize the presence of pathogens via PRRs such as TLRs. Here we have uncovered a connection between the two systems, TLR signalling and autophagy, thus linking pathogen recognition via PRRs and pathogen elimination through autophagy. Specifically, we found that ssRNA can activate TLR7 and induce autophagy in RAW 264.7 cells as a mechanism that can eliminate a non-cognate intracellular pathogen.

On the basis of multiple assays, a subset of TLR ligands can induce autophagy in murine macrophages, poly(I:C) (TLR3), LPS (TLR4) and ssRNA (TLR7). The strongest autophagy induction was observed with TLR7 ligands. Both ssRNA and imiquimod induced autophagy through TLR7. Showing a partially overlapping specificity pattern, the synthetic imidazoquinoline compound imiquimod activates both human TLR7 (hTLR7) and mouse TLR7 (mTLR7), but not human TLR8 (hTLR8) (Heil *et al*, 2003; Lee *et al*, 2003). In a partial contrast, GU-rich ssRNA is a ligand for mTLR7 and hTLR8, but not for hTLR7 in certain cells (Heil *et al*, 2004) or murine TLR8 (mTLR8), with mTLR8 responsive only to a combination of PAMPs (Gorden *et al*, 2006). Consistent with this reactivity pattern, we found that a marker of autophagy was induced in a TLR8-dependent manner when HeLa cells were infected with HIV, a known source of naturally occurring GU-rich ssRNA (Heil *et al*, 2004; Beignon *et al*, 2005; Schlaepfer *et al*, 2006; Meier *et al*, 2007). TLR7 is expressed in macrophages (Figure 5C), but its highest expression and its most prominent role are in plasmocytoid dendritic cells (pDCs) (Lee and Kim, 2007). Recently, it has been reported that in pDC autophagy can deliver a viral TLR7 ligand to the intracellular compartment where TLR7 localizes, thus bringing together the ligand and the receptor to initiate signalling leading to antiviral responses (Lee *et al*, 2007). Whereas this indicates that autophagy may enhance TLR recognition of PAMPs, the converse, that is, whether TLR induce or modulate autophagy, had not been addressed. Our present study indicates that autophagy may not be simply a peripheral pathway adding to TLR signalling, but that it could instead represent a previously unappreciated effector of TLR signalling. In support of this conclusion is a report that appeared while our study was in revision, indicating that TLR4 induces autophagy (Xu *et al*, 2007).

Although our screen has been focused on individual PAMPs, our own study with complex PAMP sources (e.g., zymosan) indicates that combinations of ligands may provoke autophagy in cases where individual PAMPs do not. Thus, we cannot rule out the possibility that Pam$_3$CSK$_4$, Pam$_2$CSK$_4$, bacterial flagellin or CpG oligonucleotide, the ligands that individually did not stimulate autophagy in our screen, can induce autophagy in certain cell types or in combination with additional inputs. A trivial explanation that the above ligands for TLR1/TLR2, TLR2/TLR6 and TLR9 when tested in our system simply did not activate the cells was experimentally ruled out, as these stimuli had the capacity to cause IκB-α degradation, activate NF-κB, and stimulate JNK phosphorylation. A different interpretation may apply to flagellin, a TLR5-specific ligand, as it also failed to activate NF-κB and did not induce JNK phosphorylation. It has been reported that RAW 264.7 macrophages do not express TLR5 and therefore do not respond to flagellin (Mizel *et al*, 2003). Although we detected TLR5 protein by western blotting (Supplementary

Figure S2C), it is unlikely that TLR5 is functional in RAW 264.7 cells.

Can rules regarding which TLR is likely to induce autophagy be inferred from the downstream factors engaged by a specific TLR? TLR3 signalling is TRIF-dependent and MyD88-independent, TLR4 can be MyD88- or TRIF-dependent, whereas TLR7 is strictly MyD88-dependent (Lee and Kim, 2007). Thus, a simple correlation between the downstream signalling pathways induced by these TLRs cannot be drawn. This is further evidenced by the finding that not all MyD88-engaging TLRs (e.g., TLR9) induced autophagy in our study. A related question is how TLRs might induce autophagy. TLRs initiate common NF-κB/mitogen-activate protein kinase (MAPK) (extracellular signal-regulated kinase (ERK), p38 and JNK) and distinct IRF3/7 pathways to coordinate innate immunity and initiate adaptive immunity against diverse pathogens (Lee and Kim, 2007). TLR7 signalling can lead to MAPK activation (ERK, JNK and p38; Heil *et al*, 2003; Lund *et al*, 2004; Koziczak-Holbro *et al*, 2007). ERK and p38 activation has been implicated in autophagy, both as a positive and a negative factor, affecting the maturation step (Corcelle *et al*, 2006, 2007). A potential role for JNK in activating autophagy via modulation of Bcl-2–Beclin 1 interactions and Beclin 1 activity (Pattingre *et al*, 2005) cannot be excluded, despite our findings that JNK phosphorylation signal is of short duration and does not differentiate between autophagy-inducing and autophagy-non-inducing PAMPs at the time periods examined. In considering MAPK, there could be a connection between p38 activation and generation of reactive oxygen species (ROS) by NADPH oxidase activation (Laroux *et al*, 2005). It is known in the case of tumour necrosis factor-α that it induces autophagy via generation of ROS (Djavaheri-Mergny *et al*, 2006), while ROS have been implicated in induction of autophagy via Atg4 (Scherz-Shouval *et al*, 2007). Furthermore, interleukin-1 receptor-associated kinase-4, which is a key downstream kinase activated upon TLR7 stimulation (Koziczak-Holbro *et al*, 2007), phosphorylates p47^{phox} and activates NADPH oxidase to generate ROS (Pacquelet *et al*, 2007). The likely relevance of ROS in autophagy induction is further underscored by our findings that zymosan (Supplementary Figure S3), in contrast to individual lipopeptides, stimulates LC3 puncta formation. Zymosan represents yeast cell wall particles that activate TLR2/TLR6, which induce inflammatory signalling, and Dectin-1, which induces phagocytosis and ROS generation (Underhill, 2003).

Among the alternative possibilities for TLR7 activation of autophagy are (a) induction of type I interferon (IFN-α and β) and (b) NF-κB activation. A pivotal role for type I interferon is unlikely as (i) RAW 264.7 macrophages stimulated with ssRNA for 4 h did not secrete IFN-β (Supplementary Figure S2B); (ii) it has been reported that IFN-α/β does not induce autophagy in RAW 264.7 macrophages (Gutierrez *et al*, 2004) and (iii) in experiments shown in Figure 1A and B, cells stimulated with poly(I:C) secreted 7–8 times more IFN-β than those stimulated with ssRNA (Supplementary Figure S2B), in inverse correlation relative to the more prominent autophagy induction with ssRNA than with poly(I:C). NF-κB activation downstream of TLR stimulation may more likely play an inhibitory role in autophagy regulation. A recent report has indicated that activation of NF-κB counteracts and represses autophagy (Djavaheri-Mergny *et al*, 2006). It is possible that

robust NF-κB activation or modulation of some other factors may preclude efficient autophagy activation by TLR7 in some cells (Z Zhao and H Virgin, personal communication). Furthermore, several TLR ligands (Pam$_3$CSK$_4$, Pam$_2$CSK$_4$, CpG) caused IκB-α degradation (Supplementary Figure S2D) and activated NF-κB (Supplementary Figure S2A), but did not induce autophagy in our study.

The connections between TLR signalling and induction of autophagy shown here link two broad aspects of innate immunity, TLR signalling and autophagy. In addition, the relationships uncovered in this work open the possibility of putting them to practical use. The TLR7 ligand imiquimod is a prescription medication known under the trade name Aldara with therapeutic applications in patients with viral infections and certain cancers (Beutner *et al*, 1998; Edwards *et al*, 1998; Syed *et al*, 1998). Our data show that activation of macrophages with imiquimod reduces mycobacterial viability in infected macrophages. This suggests a potential application of the relationships shown in this work as a basis for treatment of early or latent *M. tuberculosis* infections. The link between TLR signalling and autophagy may prove to be an unanticipated but valuable application of the detailed knowledge of TLR-signalling pathways, now expanded to the elimination of pathogens through induction of autophagy.

Materials and methods

Cell and bacterial cultures
Murine RAW 264.7 and J774 macrophage cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (complete media). HeLa cells were maintained in DMEM, 10% FBS. BMMs were derived from C57/BL6 mice or GFP–LC3 mice (Dr N Mizushima, Japan) as described previously (Via *et al*, 1998). *M. tuberculosis* var. *bovis* BCG was grown in Middlebrook 7H9 broth with 0.5% Tween, 0.2% glycerol and albumin–dextrose–catalase (ADC) supplement (BD Diagnostics, Franklin Lakes, NJ, USA) and homogenized to generate a single-cell suspension, or on 7H11 plates with 0.5% Tween, 0.2% glycerol and ADC.

Antibodies, TLR ligands, drugs, cytokines, siRNAs and DNA constructs
The rabbit polyclonal antibody against LC3 (T Ueno and E Kominami, Japan) was used at 1:500 dilution; mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rabbit polyclonal anti-TLR7, rabbit polyclonal anti-MyD88, rabbit polyclonal anti-GFP, mouse monoclonal anti-TLR5 antibodies and mouse monoclonal anti-actin antibodies were from Abcam Inc. (Cambridge, MA, USA); goat polyclonal anti-Beclin 1 antibody (Santa Cruz Biotechnology Inc., CA, USA) was used at 1:200 dilution; mouse monoclonal anti-IκB-α, rabbit polyclonal anti-phospho-JNK and rabbit polyclonal anti-JNK antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA); rabbit polyclonal anti-Atg5 was from Novus Biologicals (Littleton, CO, USA); anti-rabbit Alexa-488-conjugated antibody was from Molecular Probes (Eugene, OR, USA). Lipopeptides Pam$_2$CSK$_4$ and Pam$_3$CSK$_4$, zymosan, polyinosine–polycytidylic acid poly(I:C), imiquimod (R837), ssRNA (ssRNA40/LyoVec) and the CpG oligonocleotide ODN1826 were from InvivoGen (San Diego, CA, USA). Mouse IFN-γ, LPS from *Escherichia coli* 026:B6, E-64d, pepstatin A, rapamycin and rabbit polyclonal anti-LC3B antibody were from Sigma-Aldrich (St Louis, MO, USA). Bafilomycin A$_1$ was from LC Laboratories (Worburn, MA, USA). Secondary horseradish peroxidase-conjugated antibodies were from Pierce (Rockford, IL, USA). Control siRNA (siCONTROL non-targeting siRNA) and siRNAs for mouse LC3B, mouse Beclin 1, mouse Atg5, mouse TLR7, mouse MyD88 and human TLR8 (siGENOME SMARTpool siRNA) were from Dharmacon (Chicago, IL, USA). NF-κB-responsive luciferase reporter plasmid PathDetect® NF-κB *cis*-Reporting System was from Stratagene (La Jolla, CA, USA) and the β-galactosidase construct, pEF1-Bos, was from G Nunez (University of Michigan).

Methods
All other materials and methods are described in Supplementary data.

Supplementary data
Supplementary data are available at The *EMBO Journal* Online (http://www.embojournal.org).

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