Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion

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Autophagy defends the mammalian cytosol against bacterial infection\textsuperscript{1–3}. Efficient pathogen engulfment is mediated by cargo-selecting autophagy adaptors that rely on unidentified pattern-recognition or danger receptors to label invading pathogens as autophagy cargo, typically by polyubiquitin coating\textsuperscript{4–9}. Here we show in human cells that galectin 8 (also known as LGALS8), a cytosolic lectin, is a danger receptor that restricts \textit{Salmonella} proliferation. Galectin 8 monitors endosomal and lysosomal integrity and detects bacterial invasion by binding host glycans exposed on damaged \textit{Salmonella}-containing vacuoles. By recruiting NDP52 (also known as CALCOCO2), galectin 8 activates antibacterial autophagy. Galectin-8-dependent recruitment of NDP52 to \textit{Salmonella}-containing vesicles is transient and followed by ubiquitin-dependent NDP52 recruitment. Because galectin 8 also detects sterile damage to endosomes or lysosomes, as well as invasion by \textit{Listeria} or \textit{Shigella}, we suggest that galectin 8 serves as a versatile receptor for vesicle-damaging pathogens. Our results illustrate how cells deploy the danger receptor galectin 8 to combat infection by monitoring endosomal and lysosomal integrity on the basis of the specific lack of complex carbohydrates in the cytosol.

Galectins are \(\beta\)-galactoside-binding lectins that accumulate in the cytosol before being secreted via a leader-peptide-independent pathway\textsuperscript{10,11}. The best-characterized functions of galectins are performed extracellularly, where they bind glycans to modulate cellular behaviour. However, the occurrence of galectins in the cytosol, which under physiological conditions is devoid of complex carbohydrates, makes them prime candidates for a role as danger and/or pattern-recognition receptors. Galectin 3 (also known as LGALS3) accumulates on damaged physiological conditions is devoid of complex carbohydrates, makes them prime candidates for a role as danger and/or pattern-recognition receptors. Galectin 3 (also known as LGALS3) accumulates on damaged vesicles, although the functional consequences of its recruitment remain unknown\textsuperscript{12,13}. We screened a panel of human galectins for their ability to detect invasion by \textit{Salmonella enterica} serovar Typhimurium. At 1 h post-infection (p.i.), galectin 3, 8 and 9 accumulated on about 10% of intracellular \textit{S. Typhimurium} (Fig. 1a, b and Supplementary Fig. 1a), of which 90% were associated with LAMP1 (Supplementary Fig. 1b). Recruitment of galectins peaked between 1 h and 2 h p.i. (Supplementary Fig. 1c). As galectin 3, 8 and 9 were recruited to \textit{Salmonella}-containing vesicles (SCVs), we used short interfering RNAs (siRNAs) to test whether their depletion causes hyperproliferation of \textit{S. Typhimurium}. Cells lacking galectin 8 or NDP52, but not galectin 3 and/or 9, failed to suppress proliferation of \textit{S. Typhimurium} (Fig. 1c and Supplementary Figs 2a–c, and 3a). Microscopic analysis confirmed that the greater bacterial burden of cells lacking galectin 8 was caused by enhanced proliferation rather than differential uptake of bacteria (Supplementary Fig. 3b). Hyperproliferating bacteria in cells lacking galectin 8 appeared mainly in a LAMP1-negative compartment (Supplementary Fig. 3c), consistent with colonization of the cytosol. We conclude that galectin 8 is an antibacterial restriction factor.

As autophagy provides antibacterial protection to cells, the decoration of SCVs with galectins might be an autophagy-inducing signal, analogous to ubiquitin coating\textsuperscript{14,15}. We therefore tested binding of galectins to autophagy receptors that restrict the proliferation of \textit{S. Typhimurium}, that is, NDP52, p62 and optineurin\textsuperscript{5–9}. We found in a luminescence-based mammalian interactome mapping (LUMIER) assay that galectin 8 and NDP52 interacted specifically (Fig. 2a and Supplementary Fig. 4a). Binding was confirmed by precipitating endogenous NDP52 with Flag-tagged galectin 8 (Fig. 2b). SCVs double labelled by endogenous galectin 8 and NDP52 were prominent in \textit{Salmonella}-infected HeLa cells (Fig. 2c). In cells expressing yellow fluorescent protein (YFP)-tagged galectins the majority of

**Figure 1 | Galectin 8 responds to infection by \textit{S. Typhimurium} and restricts bacterial proliferation.** a, Analysis of HeLa cells stably expressing YFP fused to the indicated galectins and infected with \textit{S. Typhimurium} for 1 h. a, Percentage of bacteria coated by the indicated galectins. YFP-positive bacteria were counted by microscopy. Mean and standard deviation (s.d.) of triplicate HeLa cultures, \(n > 100\) bacteria per coverslip. b, Confocal micrographs. Arrowheads, bacteria shown in insets. DAPI, 4',6-diamidino-2-phenylindole. c, Kinetics of fold replication for \textit{S. Typhimurium} in HeLa cells transfected with the indicated siRNAs. Bacteria were counted on the basis of their ability to form colonies on agar plates. Mean and s.d. of triplicate HeLa cultures and duplicate colony counts. siRNAs are further characterized in Supplementary Fig. 2a–c. **\(P < 0.01\), Student’s t-test. Scale bar, 10 \(\mu\)m.

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galectin-positive SCVs had accumulated NDP52 (Fig. 2d and Supplementary Fig. 5a). Furthermore, at 1 h p.i. NDP52 and galectin 8 co-localized tightly in a pattern distinct from p62 or ubiquitin “microdomains” (Supplementary Fig. 5b, c).

To characterize further the interaction between galectin 8 and NDP52 we determined their respective binding sites. Galectin 8 contains two carbohydrate-recognition domains (CRD) (Supplementary Fig. 6a). NDP52 bound galectin-884,877 (that is, amino acids 229–360), equivalent to the second CRD, but not galectin-88,877 (that is, amino acids 1–228) (Supplementary Fig. 6b). NDP52 harbours a SKICH domain, a coiled coils-forming region, and a ubiquitin-binding zinc finger (Supplementary Fig. 6a). Galectin 8 bound NDP5281,370 but not NDP529,370 (Supplementary Fig. 6c). The NDP52 fragment spanning residues 370–393 is therefore essential for binding galectin 8. This fragment, as well as NDP5285,380, purified as GST-fusion proteins, bound galectin 8 (Supplementary Fig. 6d). A point mutation within NDP5285,380 (L374A) abrogated binding to galectin 8, without compromising binding to ubiquitin when introduced into full-length NDP52 (Supplementary Fig. 6d, e). Binding of galectin 8 to NDP52 is direct, as the purified proteins interacted (Supplementary Fig. 6f).

To determine whether one monomer of the NDP52–galectin-8 heteromeric complex recruits the other partner, the accumulation of galectins on SCVs in cells depleted of NDP52 or TBK1 was analysed (Fig. 3a and Supplementary Fig. 2). Galectin 3, 8 and 9 re-distributed on SCVs if galectin 1, suggesting compartment-specific differences in the distribution of galectin ligands. GPN failed to induce speckles of galectin-8(R232H), galectin-8(R69H) did not accumulate at SCVs, showing that the amino-terminal CRD is required for carbohydrate-dependent recruitment of galectin 8 to SCVs (Fig. 3c). To test whether the carbohydrates detected by galectin 8 are of microbial origin, binding of recombinant galectin 8 to bacteria in vitro was analysed. Galectin 8 did not bind to S. Typhimurium but stained blood-group-B-positive bacteria (Escherichia coli strain O86) (Fig. 3d), suggesting that galectin 8, when accumulating on SCVs, recognizes host glycans. The occurrence of galectin-8 ligands in host cells was confirmed by staining HeLa cells with recombinant galectin 8 (Fig. 3d). Direct evidence that host glycans recruit galectins to SCVs was obtained from experiments with CHO-Lec3.2.8.1 cells, which lack mature glycans and in which recruitment of galectins to SCVs was severely impaired (Fig. 3e). The detection of host glycans on damaged vesicles by galectin 8 suggests that it is not a receptor specific for S. Typhimurium. We therefore tested whether sterile damage to vesicles is detected by galectins. Osmotic damage of endosomes induced dense puncta formed by galectin 3, 8 and 9 but not by galectin 1 (Fig. 3f and Supplementary Fig. 8). Damage to lysosomes by glycy1-phenylalanine 2-naphthylamide (GPN) treatment resulted in the initial loss of lysotracker staining, followed by the appearance of galectin 3, 8 and 9 speckles (Supplementary Fig. 9a). In contrast to damaged SCVs and endosomes, burst lysosomes were also detected by galectin 1, suggesting compartment-specific differences in the distribution of galectin ligands. GPN failed to induce speckles of galectin-8(R69H) (Supplementary Fig. 9b), thereby indicating that binding of glycans to the N-terminal CRD of galectin 8 is required to detect lysosomal damage. The capacity of galectin 3, 8 and 9 to detect vesicle damage by binding exposed host glycans suggests their ability to sense the invasion of cells by a wide range of vesicle-damaging pathogens. Indeed, galectin 3, 8 and 9 also accumulated around Gram-positive Listeria monocytogenes and Gram-negative Shigella flexneri (Supplementary Fig. 10), proving that these galectins detect the invasion of cells by phylogenetically distant bacteria. We conclude that galectin 3, 8 and 9 are danger receptors that sense the exposure of host glycans on ruptured membranes and thereby monitor the integrity of the endosomal/lysosomal compartment.

Figure 2 | Galectin 8 binds NDP52. a, LUMIER binding assay: normalized ratio between luciferase activity bound to beads and present in lysates. Lysates of 293ET cells expressing NDP52, p62 or optineurin each fused to Flag and the indicated Flag-tagged galectins were incubated with anti-Flag beads. Flag-tagged proteins are further characterized in Supplementary Fig. 4a. b, Lysates of 293ET cells, expressing Flag-tagged proteins as indicated, were immunoprecipitated with anti-Flag beads. Lysates and immunoprecipitates (IP) were blotted for the presence of Flag-tagged proteins and endogenous NDP52. c, Confocal images of HeLa cells infected with S. Typhimurium for 1 h and stained with antisera against NDP52 and galectin 8. Arrowheads, bacteria shown in insets. d, Co-localization of NDP52 with galectin-positive bacteria in HeLa cells stably expressing YFP fused to the indicated galectins, infected with S. Typhimurium and stained with NDP52 antisera 1 h after infection. Mean and s.d. of duplicate HeLa cultures, n > 100 bacteria per coverslip, representative of two independent experiments. Scale bar, 10 μm.
Galectin 8 is a danger receptor that senses cytosolic host glycans and recruits NDP52 to restrict Salmonella proliferation. a, Percentage of S. Typhimurium coated by the indicated galectins. HeLa cells stably expressing YFP-tagged galectins were counted by microscopy at 1 h p.i. siRNAs were further characterized in a pairwise co-localization of NDP52 with galectin-8- and LC3-positive bacteria (Fig. 2d and Supplementary Fig. 11). We then tested whether depletion of galectin 8 impairs autophagy of S. Typhimurium. In the absence of galectin 8 fewer bacteria were targeted by LC3 (Fig. 4b) and of the remaining LC3-positive bacteria fewer had accumulated NDP52 (Supplementary Fig. 11). In contrast, galectin-8 recruitment to SCVs did not require autophagy as it occurred undisturbed in ATG5−/− fibroblasts (Supplementary Fig. 12). We conclude that the danger receptor galectin 8, by recruiting NDP52, directs autophagy towards invading bacteria.

The recruitment of NDP52 to invading bacteria is mediated by two signals, the newly discovered carbohydrate-dependent galectin-8 pathway and the previously known ubiquitin-dependent pathway. Their differential contribution to the recruitment of NDP52 to S. Typhimurium was investigated by analysing NDP52 mutants selectively disabled to bind galectin 8 and/or ubiquitin. For accurate scoring,
NDP52ASKICH was used, as this truncated allele is distributed diffusely throughout the cytosol. NDP52127–446, associated with SCVs at all time points investigated (Fig. 4c). Deleting the carboxy-terminal ubiquitin-binding zinc finger (NDP52127–420) impaired the recruitment of NDP52 to S. Typhimurium at late but not early time points. In contrast, NDP52127–446(L374A), which lacks affinity for galectin 8 but not ubiquitin (Supplementary Fig. 6d), did not co-localize with bacteria at 1 h p.i., but accumulated progressively over time (Fig. 4c). Direct evidence for early galectin-8-dependent and late galectin-8-independent recruitment of NDP52 to S. Typhimurium was obtained from cells depleted of galectin 8, in which NDP52 and bacteria co-localized at 4 h but not at 1 h p.i. (Fig. 4e). NDP52127–420(L374A), deficient in binding to ubiquitin and galectin 8, did not translocate to SCVs at any time point tested (Fig. 4c). Taken together, NDP52 relocates to SCVs in response to two signals, which are active against bacteria at different stages of invasion. The early response to invading bacteria requires the galectin-8–dependent pathway, whereas the zinc-finger-dependent pathway dominates at later time points.

The galectin-8/NDP52 pathway sheds light on why most intracellular bacteria avoid the cytosol and prefer vesicular compartments. The cytosol seems to be protected by synergistic layers of antibacterial defence that activate autophagy at distinct steps of the invasion process. An early line of defence comprises the accumulation of diacylglycerol on bacteria-containing vesicles, which subsequently become the target of autophagy19. Bacteria escaping the diacylglycerol pathway and exposing host glycans on their damaged vacuoles are targeted by galectin 8 and NDP52, as described in this work. A third layer of defence coats invading bacteria with polyubiquitin6–10. Neither the enzymatic machinery for nor the substrate of ubiquitination have been identified, although LRSAM1, a RING-finger E3 ubiquitin ligase, contributes to autophagy of S. Typhimurium21. Peptidoglycan and septin cages surrounding cytotoxic bacteria also contribute to autophagy22–25. Defects in this intricate network of autophagy-inducing defence pathways are likely to cause susceptibility to infection and promote inflammation, for example in Crohn’s disease26–28. Galectin 8 is positioned strategically at the cellular entry point for a variety of pathogens and is therefore expected to have shaped pathogen evolution.

**METHODS SUMMARY**

Galectins were cloned as YFP fusions and transduced into HeLa cells. HeLa cells were infected with S. Typhimurium strain 12023. For confocal microscopy cells were fixed in paraformaldehyde. Bacterial growth was assessed by a gentamycin protection assay. Knockdowns were accomplished with Stealth siRNAs. LUMIER assays were performed as described10. For flow-cytometric analysis samples were incubated with lysates of E. coli expressing His-GST fusion proteins, followed by anti-His antibody and goat anti-mouse serum. Statistical testing was performed using two-tailed Student’s t-test.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Antibodies. Antibodies were from QIAGEN (Penta-His), the Developmental Studies Hybridoma Bank (LAMP1), BD Transduction Laboratories (p62), Santa Cruz (GAL8-H80, TBK1-C100), R&D Systems (galectin 8), Transduction Laboratories (NDP52, for western blots), Enzo Life Science (ubiquitin FK2), Sigma (ATGS, Flag M2), Dabcyl (HRR-conjugated reagents), Jackson Immunoresearch Laboratories (goat anti-mouse-phycocerythrin (PE)) and Invitrogen (Alexa-conjugated anti-mouse and anti-rabbit antisera). The antiserum against NDP52 used for immunofluorescence was a gift from J. Kendrick-Jones.

Plasmids. MSP or closely related plasmids were used to produce recombinant MLV for the expression of proteins in mammalian cells35. pETM plasmids were gifts from A. Geerlof. Open reading frames encoding human galectins, NDP52, p62, optineurin, ubiquitin, ATG5 and LC3C were amplified by PCR or have been described8,32. Mutations were generated by PCR and verified by sequencing.

Bacteria. S. Typhimurium (strain 12023), provided by M. Barrios-Rodiles, was grown overnight in Luria broth (LB) and sub-cultured (1:33) in fresh LB for 3.5 h before infection. HeLa cells in 24-well plates were infected with 20 μl of such cultures for 15 min at 37°C. Following two washes with warm PBS and an incubation with 100 μg ml⁻¹ gentamycin for 2 h cells were cultured in 20 μg ml⁻¹ gentamycin. To enumerate intracellular bacteria, cells from triplicate wells were lysed in 1 ml cold PBS containing 0.1% Triton-X-100. Serial dilutions were plated in duplicate on TYE agar.

L. monocytogenes strain EGD (BUG 600), provided by C. Tang, was grown overnight in Brain Heart Infusion (BHI) at 30°C with shaking. Five-hundred microlitres of diluted cultures (1:133) were added to HeLa cells in 24-well plates. Samples were centrifuged for 10 min at 670 g and incubated in 37°C for 30 min, cells were washed with warm PBS and cultured and in 100 μg ml⁻¹ gentamycin for 2 h and 20 μg ml⁻¹ thereafter.

S. flexneri M90T, provided by C. Tang, was grown overnight in Tryptic Soy Broth (TSB) and sub-cultured (1:100) in fresh TSB for 2 h before infection. Bacteria were resuspended in warm IMDM and 100 μl were added to HeLa cells in 24-well plates. Samples were centrifuged for 10 min at 670 g. Following incubation at 37°C for 30 min, cells were washed with warm PBS and cultured in 100 μg ml⁻¹ gentamycin for the next hour and 20 μg ml⁻¹ gentamycin thereafter.

Cell culture. Cells were grown in IMDM supplemented with 10% FCS at 37°C in 5% CO₂. HeLa cells were obtained from the European Collection of Cell Cultures, CHO and Lec3.2.8.1 cells43 were obtained from from P. Stanley, ATGS / MEFs33 from N. Mizushima.

RNA interference. 5 × 10⁴ cells per well were seeded in 24-well plates. The following day, cells were transfected with 40 pmol of siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen) in OptiMem medium (Invitrogen). OptiMem was replaced with complete IMDM medium after 4 h and experiments were performed after 3 days. siRNAs targeted the following sequences: siNDP52 5'-UUCAGUGAAGACGGCGUCUGCUCCU-UU; siGAL8 #36 5'-GAAACAAGAGGUGUGGCGAGUCAA-UU; siGAL8 #38 5'-GGACAAUCCAGGUGGCGAGUCAA-UU; siGAL9 #807 5'-UGUGCAACAGGAGCGAGAAGGGG-GG; siTBK1 5'-GACAGGAAUGUGUGAUCACAC(TT)₃₄.

To render galectin 8 resistant to siGAL8 #38, silent mutations (underlined) GGAATAGTTTCAGTGCCGATTAA were introduced by PCR and confirmed by sequencing.

Immunoprecipitation and western blot. Post-nuclear supernatants from 2 × 10⁶ HeLa cells expressing Flag-tagged proteins were obtained following lysis (150 mM NaCl, 0.1% Triton-X-100, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA and protease inhibitors). Protein complexes were immunoprecipitated for 2 h with Flag agaore before washing. Samples were eluted with Flag peptide and separated on 4–12% denaturing Bis-Tris gels (Invitrogen). Visualization following immunoblotting was performed using ECL detection reagents (Amersham Bioscience).

LUMIER assays. LUMIER binding assays30,32 with pairs of putative interactors, one fused to luciferase and the other fused to GST or Flag, were performed in LUMIER lysis buffer (150 mM NaCl, 0.1% Triton-X-100, 20 mM Tris-HCl (pH 7.4), 5% glycerol, 5 mM EDTA and proteinase inhibitors). GST-fusion proteins were immobilized on beads before incubation with the luciferase tagged binding partner for 2 h. For Flag-based assays, both proteins were expressed in 293T cells and immobilized using Flag-agarose. After washing in lysis buffer, proteins were eluted with glutathione or Flag peptide in Renilla lysis buffer (Promega). Relative luciferase activity represents the ratio of activity eluted from beads and present in lysates.

FACS. To examine the binding of galectin 8, bacteria in stationary phase or HeLa cells were washed in PBSF (PBS, 2% FCS) and incubated for 30 min at 4°C with cleared lysates of E. coli expressing His-GST fusion proteins, followed by incubations with anti-His antibody and PE-conjugated goat anti-mouse serum. Bacteria were fixed in 4% paraformaldehyde before analysis.

Sterile damage to vesicles. Endosomes were lysed by exposing cells for 10 min to hypertonic medium (0.5 M sucrose in PBS, with or without 10% PEG10000), followed by two PBS washes and an incubation in 60% PBS for 3 min44. Cells were returned to complete medium for 20 min, before being fixed in paraformaldehyde. For live imaging of lysosomal damage, cells were labelled for 1 h with 100 nM LysoTracker Red (Invitrogen), washed with PBS, incubated in Leibovitz L15 medium and, after acquisition of the first image, exposed to 333 μM GPN45.

Microscopy. HeLa cells were grown on glass cover slips before infection. After infection, cells were washed twice with warm PBS and fixed in 4% paraformaldehyde in PBS for 30 min. Cells were washed twice in PBS and then quenched with PBS pH 7.4 containing 1 M glycine and 0.1% Triton-X-100 for 30 min before blocking for 30 min in PBTB (PBS, 0.1% Triton-X-100, 2% BSA). Cover slips were incubated with primary followed by secondary antibodies for 1 h in PBTB before being mounting in medium containing DAPI (Vector Laboratories). At least 100 events per slide were scored in quantitative assays. Confocal images were taken with a ×63, 1.4 numerical aperture objective on either a Zeiss 710 or a Zeiss 780 microscope. Live imaging was performed on a Nikon Eclipse Ti equipped with an Andor Revolution XD system and a Yokogawa CSU-X1 spinning disk unit.

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