Galectin-3, a marker for vacuole lysis by invasive pathogens

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Summary

Shigella bacteria invade macrophages and epithelial cells and following internalization lyse the phagosome and escape to the cytoplasm. Galectin-3, an abundant protein in macrophages and epithelial cells, belongs to a family of beta-galactoside-binding proteins, the galectins, with many proposed functions in immune response, development, differentiation, cancer and infection. Galectins are synthesized as cytosolic proteins and following non-classical secretion bind extracellular beta-galactosides. Here we analysed the localization of galectin-3 following entry of Shigella into the cytosol and detected a striking phenomenon. Very shortly after bacterial invasion, intracellular galectin-3 accumulated in structures in vicinity to internalized bacteria. By using immuno-electron microscopy analysis we identified galectin-3 in membranes localized in the phagosome and in tubules and vesicles that derive from the endocytic pathway. We also demonstrated that the binding of galectin-3 to host N-acetyllactosamine-containing glycans, was required for forming the structures. Accumulation of the structures was a type three secretion system-dependent process. More specifically, existence of structures was strictly dependent upon lysis of the phagocytic vacuole and could be shown also by Gram-positive Listeria and Salmonella sifA mutant. We suggest that galectin-3-containing structures may serve as a potential novel tool to spot vacuole lysis.

Introduction

Invading pathogens need to evade the immune system in order to maintain replication within the host. Shigella serves as an example of such a pathogen whereas bacterial invasion is mediated by the type three secretion system (TTSS) that is activated upon contact with the host cell and injects effector proteins to the cell cytoplasm (Enninga et al., 2005). Following internalization, bacteria lyse the phagosome by TTSS activity. This process is poorly understood due to the lack of reliable markers for vacuole lysis, but is a critical step to the bacteria that can avoid phagosome-lysosome fusion and start replicating in the cytoplasm and spreading from cell to cell. At the same time it is a turning point to the host initiating a response following the sensing of a foreign entity inside the cell. This response is initiated as signalling occurring already at the phagosome, e.g. by intracellular receptors such as Nods leading to the activation of inflammatory gene expression (reviewed in Herskovits et al., 2007; Phalipon and Sansonetti, 2007; Kufer et al., 2008; Schroeder and Hilbi, 2008). In an attempt to analyse this inflammatory cellular program following infection, we compared the transcriptome of the intestine of infected and non-infected newborn mice. Interestingly, one protein induced after...
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Shigella flexneri infection was galectin-3 (T. Pedron and P. Sansonetti, unpublished).

Galectin-3, also known as Mac-2, is a member of an ancient protein family, the galectins, defined by conserved sequence elements and affinity for β-galactose-containing carbohydrates (Houzelstein et al., 2004; Leffler et al., 2004). The galectins are small soluble proteins that, of special relevance here, are synthesized as cytosolic proteins, without a signal peptide. However, they may also be targeted to the nucleus, enter intracellular vesicles or be secreted by a non-classical (non-ER-Golgi) pathway (Hughes, 1999; Menon and Hughes, 1999; Delacour et al., 2007). Consequently, a surprising array of functional activities have been reported including nuclear, cytosolic, intravesicular and extracellular activities (Ochieng et al., 2004; Patterson et al., 2004; Ashery et al., 2006; Toscano et al., 2007; Rabinovich et al., 2007). Since the β-galactoside-containing glycoconjugates are typically not found in the cytosol or nucleus, but only in vesicles, on the cell surface and extra-cellularly, a galectin needs to cross a membrane (by non-classical secretion) to reach them. Their subsequent interaction and cross-linking has been suggested to determine targeting to specific parts of the plasma membrane (Delacour et al., 2007), residence time of specific receptors at the cell surface (Lau et al., 2007; Stanley, 2007), or extracellular transmembrane signalling that may modulate cell activation, differentiation and survival (Rabinovich et al., 2007).

On a larger functional scale, galectins were suggested to be cytokine/chemokine-like molecules (Toscano et al., 2007) and active players in the inflammatory response (Rabinovich et al., 2002; Rubinstein et al., 2004). Galectin-3 is found in many tissues, mainly in epithelial and myeloid cells and involved in many functions (Dumic et al., 2006). A proinflammatory role was suggested based on experiments in vitro and in vivo using galectin-3 null mutant mice (Colnot et al., 1998; Hsu et al., 2000, reviewed in Liu and Hsu, 2007). During infection processes, galectin-3 was suggested to be a potential PRR (Pathogen Recognition Receptor) based on its binding to certain bacterial, parasite and fungal products (Sato and Nieminen, 2004), especially those containing β-galactose but also others. Interestingly, galectin-3 was proposed to be also a potential danger signal based on its ability to be passively released upon cellular damage from many cells found at the site of infection and on its ability to be actively secreted from inflammatory macrophages (Sato and Nieminen, 2004; Liu and Hsu, 2007; McClung et al., 2007).

The induction of galectin-3, a cytosolic protein that may reach the extracellular space, by Shigella, an invasive pathogen that reaches the cytosol, raises the questions of where and how they meet. To address this, we took a cell biology approach to carefully look at galectin-3 at the cellular level following Shigella infection. We report here the rapid recruitment of the galectin-3 protein to lysed vacuoles following the invasion of both Gram-negative and -positive bacteria to the cell. Galectin-3 does not interact directly with the bacteria, but apparently with β-galactose-containing glycoconjugates of the lysed vacuole membrane.

We discuss the importance of this phenomenon to be used in further host-pathogen studies.

Results

Galectin-3 accumulates in structures in vicinity of intracellular bacteria

Galectin-3 localization was first analysed in different epithelial cell types infected or not with Shigella flexneri virulent strain, M90T (Fig. 1A). In uninfected cells, the galectin-3 protein was diffusely distributed in the nucleus and cytoplasm, as shown previously in other cell types (Dumic et al., 2006). Strikingly, upon infection of Caco-2 cells with Shigella, M90T, for 60 min, we observed a massive recruitment of galectin-3 around the intracellular bacteria (Fig. 1A). Infection of Caco-2 cells for shorter times (20–60 min) gave the same results (data not shown). The same phenomenon appeared in two other types of epithelial cells, HeLa (Fig. 1B) and MDCK (data not shown) infected with Shigella, M90T, for 30 min. Phalloidin staining of HeLa cells illustrated normal actin architecture when galectin-3 recruitment occurred. Detailed inspection of bacteria in HeLa cells showed that galectin-3 did not colocalize with bacteria, but rather localized in structures near internalized bacteria (Fig. 1B, inset). We named these structures galectin-3-containing structures (G3CS).

Since macrophages are initial targets of Shigella entry and may express high levels of galectin-3, we examined the emergence of G3CS also in this cell type. Indeed, 20 min post infection of J774 macrophages (or raw 264.7 macrophages, data not shown) we could detect G3CS (Fig. 1C). We could also demonstrate that recruitment of G3CS in vicinity to the bacteria occurred in primary bone marrow-derived macrophages (data not shown), hence not restricting the phenomenon only to macrophage cell lines. By carefully inspecting individual intracellular bacteria in various cells we could detect diverse types of G3CS that were sometimes, but not always, polar, i.e. localized to one end of the bacteria.

Galectins have in common a conserved β-galactoside binding site, but vary in fine specificity for larger saccharides due to differences in neighbouring sites (Leffler et al., 2004), which gives the vastly different affinity for natural glycoproteins as demonstrated by Cederfur et al.,
Therefore, it was of interest to see if another galectin could also be recruited to bacteria and we thus analysed the localization of galectin-7 during Shigella infection in J774 macrophages. No recruitment of this protein to bacteria was seen (data not shown), demonstrating that G3CS acquisition was not shared between these two galectins.

Time-course of G3CS acquisition in J774 macrophages

To analyse timing and kinetics of G3CS appearance, we examined a synchronized infection during 5–60 min post infection of J774 cells (see Experimental procedures). We defined a bacterium positive for G3CS if it had at least one G3CS in its close vicinity. As shown in Fig. 2A, G3CS-positive bacteria started appearing about 15 min post infection. The percentage of these bacteria increased with time post infection, it was negligible 5 min post infection (2% positive bacteria), increased to 17% 15 min post infection, reached 45% 25 min post infection and did not further change 60 min post infection. As Shigella was previously shown to cause apoptosis in macrophages (Zychlinsky et al., 1992), we wanted to verify the amount of apoptotic cells during this time-course. As shown if Fig. 2B, TUNEL staining demonstrated that the cells were not apoptotic at least 25 min post infection (note that there were no TUNEL-positive cells 15 min time post infection, except for one occasional cell marked by an arrow). We could identify apoptotic cells 60 min post infection (60 min image in Fig. 2A, which shows about 50% apoptotic cells). Thus, during 25 min, in not yet apoptotic cells, there was an active recruitment of the galectin-3 protein to about half of the population of intracellular bacteria. This result shows that the process of galectin-3 reorganization was rapid following internalization of the bacteria and not the result of an apoptotic process.

To further demonstrate the nature and evolvement of structures, we analysed the time-course samples by confocal microscopy. The images in Fig. 2C demonstrate a typical evolving process of G3CS in J774 macrophages infected with Shigella M90T. Five minutes after infection, galectin-3 localized in vesicular structures throughout the
A

B

C

Time after infection

% of gal3-positive bacteria

Negative control

Positive control

Tunel 5min

Tunel 15min

Tunel 25min

Tunel 60min

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cell with only occasional close to bacteria. Fifteen minutes after infection, G3CS started being recruited close to bacteria and 60 min post infection G3CS evolved to tubular structures sometimes reaching the size of a bacterium (arrow).

Galectin-3 protein is localized to membranes of the phagosome and the endocytic pathway

In order to better understand the nature and the possible function of intracellular galectin-3 structures beyond IF imaging, we performed immuno-electron microscopy (EM) analysis of J774 macrophages infected for 20–40 min with Shigella M90T strain. At this time point about half of the bacteria recruited G3CS (Fig. 2A). As can be seen in Fig. 3A, after 20 min infection of J774 macrophages, galectin-3 labelling was localized at the limiting membrane of bacteria containing vacuoles (phagosomes) (Fig. 3A and B, arrowheads) and in tubular structures connected to the phagosome (Fig. 3B, arrow). Figure 3C, showing cells infected for 40 min, illustrates a phagosome membrane that is less preserved along with an increased size of tubules (arrows), similar to what was seen by IF. It was not clear to us whether the labelled membranes derived from the phagosome itself or from the starting of a fusion with other cellular compartments. We thus tested if the structures derived from the endocytic pathway. To this end, we labelled the cells with BSA-gold followed by bacterial infection for 20 min. As shown in Fig. 3D and E, we could see structures labelled with BSA-gold (5 nm gold particles) and with galectin-3 (10 nm gold particles) (arrow, Fig. 3E), structures that were labelled only by the galectin-3 protein (arrows, Fig. 3D) and structures labelled by only BSA-gold (arrowheads, Fig. 3E). Our immuno-electron microscopy analysis thus shows that a subset of the G3CS derived from phagosome membranes and from tubules/vesicles intersecting with the endocytic pathway.

The lectin activity of galectin-3 and a host glycoconjugate are both required for assembly of G3CS

We next wanted to know whether the lectin activity of galectin-3 was required for the assembly of G3CS. To this end, we utilized an SKBR3 breast cancer cell line that does not express endogenous galectin-3 (Ruebel et al., 2005) and stably expressing either GFP-galectin-3 or GFP-galectin-3 R186S mutant. This mutated galectin-3 has lost its ability to bind LacNac residues, the predominant galectin-3 binding site in cell surface glycoproteins, yet the point mutation did not disrupt the structure of galectin-3 protein as the protein retained its ability to bind lactose (Cumpstey et al., 2007; Delacour et al., 2007) and also its cytosolic localization was normal (Fig. 4A). As shown in Fig. 4A, GFP-G3CS could be identified in vicinity to the bacteria showing that the GFP did not interfere with the assembly of G3CS. Unlike SKBR3 cells complemented with galectin-3-GFP showing GFP-G3CS in vicinity to the bacteria, cells complemented with GFP-galectin-3 R186S mutant did not demonstrate any G3CS (Fig. 4A). This result shows that the lectin activity of galectin-3 was essential to form G3CS. We thus assumed that a putative glycosylated molecule was required for the assembly of G3CS. To verify if it might be a host molecule, we utilized a CHO Lec1 mutated in the Mgtat1 gene that encodes β1,2-N-acetylgalactosaminyltransferase I (GlcNAc-T1). These cells fail to add galactose to N-linked saccharides, resulting in the lack of both complex and hybrid N-glycans (Robertson et al., 1978; Chaney and Stanley, 1986; Puthalakath et al., 1996; Chen and Stanley, 2003) and do not bind exogenous galectin-3 to their surface (Patnaik et al., 2006). As shown in Fig. 4B, panel i, CHO parental cells exhibited, as all other galectin-3 expressing cell types, G3CS in vicinity to internalized Shigella M90T bacteria. However, no G3CS could be identified in infected CHO Lec1 mutant cells (Fig. 4B, panel ii). Similar results were obtained with the MDCK-RCA2 cell line (data not shown), which is deficient in galactose linked to macromolecules because of a lower UDP-Gal transport rate into the Golgi apparatus (Brandli et al., 1978; Chaney and Stanley, 1988; Olczak and Guillen, 2006), resulting in deficient interaction with galectin-3 (Delacour et al., 2007). These results show that formation of G3CS following Shigella infection requires galactose-containing host N-linked glycans.

Fig. 3. Galectin-3 localizes to the phagosome and G3CS intersect with the endocytic pathway. Immuno-electron microscopy of J774 macrophages infected with S. flexneri M90T.

A–C. Macrophages were infected with S. flexneri M90T bacteria for 20 (A and B) or 40 min (C). Cells were fixed, prepared for cryosectioning and immunolabelling for galectin-3 (10 nm particles). (A and B) Label for galectin-3 is found at the limiting membrane of the M90T containing vacuole (arrowheads) but also in tubular structures, which can be connected to the limiting membrane of the vacuole (arrow in B). (C) Longer infection with M90T leads to an increase in size of the tubules (arrows), whereas the limiting membrane of the vacuole is less preserved.

D–F. Macrophages were pulsed for 30 min with BSA-gold (5 nm particles) before infection with M90T bacteria and thawed cryosections were labelled for galectin-3 (10 nm). (D) Detail from the vicinity of a M90T containing vacuole. Galectin-3 is present in electron dense tubules/vacuoles negative for BSA-gold (arrows). (E) Galectin-3 localizes to a vacuolar/tubular structure, containing also BSA-gold (arrow). Note that label for galectin-3 is absent from vacuoles, where BSA-gold accumulates (arrowheads). (F) Quantification of relative occurrence of galactin-3 in Shigella-containing vacuoles. Class 1 no galectin-3 positive structure in close vicinity of the M90T containing vacuole (but phagosome was positive for galectin-3); class 2 galectin-3 positive but BSA-gold negative structure close to the vacuole (as shown in D); class 3 galectin-3 and BSA-gold positive structure close to the vacuole (see E). Bars = 200 nm.

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Type three secretion system is required for the acquisition of G3CS

We hypothesized that a bacterial molecule could be involved in recruiting the G3CS. We assumed it might be lipopolysaccharide (LPS) as it was previously shown that galectin-3 could bind LPS of Pseudomonas aeruginosa, Klebsiella pneumoniae and Salmonella minnesota R7 (Mey et al., 1996; Gupta et al., 1997). However, several correlative data argued against the involvement of LPS in G3CS recruitment: (i) the same G3CS appeared following infection with a galU mutant, which does not possess any LPS O antigen repetitive subunits (Sandlin et al., 1995); (ii) recombinant galectin-3 did not show binding to Shigella in vitro; and (iii) no significant colocalization between LPS and galectin-3 was observed in EM double immuno-labelling for galectin-3 and LPS (data not shown). In order to verify if there was a bacterial effector implicated in the formation of G3CS, we tested the ability of a non-virulent strain of Shigella, BS176, lacking the virulence plasmid, to recruit intracellular G3CS. As this strain is non-invasive in epithelial cells, we took an advantage of the phagocytic capacity of J774 macrophages and tested this strain for acquiring G3CS in these cells. As can be seen in Fig. 5A, we could not observe any G3CS in the vicinity of the non-virulent Shigella strain. In order to verify which protein encoded by the virulence plasmid was required for the recruitment of G3CS, we tested the ability of nine different Shigella mutants to acquire the structures: mxiE, a transcription factor mutant, icsB, ospF, icsA and ipgD, mutants involved in various host signalling pathways and mxiD, ipaB, ipaC and ipaD, mutants involved in the TTSS assembly (Schroeder and Hilbi, 2008). All the first five mutants demonstrated the same level of recruited G3CS in infected cells (data not shown). The four mutants deficient in the TTSS did not show any G3CS recruitment following infection (Fig. 5B and data not shown). This group included mxiD mutant that did not have any functional TTSS, ipaD mutant impaired in secretion of IpaB and IpaC and ipaB or ipaC mutants required for the formation of the translocon inserted in the host membrane. Rescue of ipaB and ipaC mutants with plasmids expressing IpaB or IpaC, respectively, restored the recruitment of G3CS to infected cells (Fig. 5B). Thus, these results show that acquisition the G3CS was dependent upon having a functional TTSS along with its translocon. Furthermore, our data suggest that it was rather the activity of the entire TTSS apparatus rather than a single effector triggering for the formation of G3CS.

G3CS are recruited during vacuole lysis in J774 macrophages

Effectors encoded by the TTSS of Shigella are required both for the process of invasion and vacuole lysis (Maurrelli et al., 1985; Page et al., 1999; Schuch et al., 1999). We thus wondered whether it was invasion or vacuole lysis step in which the TTSS activation was required for acquiring the G3CS. To discriminate between these two possibilities, we wanted to uncouple invasion and vacuole lysis. To this end, we utilized an ipaC null mutant recomplemented by recombinant IpaC containing a fragment of 14 amino acids inserted between residues N351 and S352 (Barzu et al., 1997). This ipaC/IpaC351 strain was shown to be non-invasive in epithelial cells and yet retained the ability to lyse the vacuole when phagocytosed by macrophages (Barzu et al., 1997). As shown in infected cells (data not shown). The four mutants deficient in the TTSS did not show any G3CS recruitment following infection (Fig. 5B and data not shown). This group included mxiD mutant that did not have any functional TTSS, ipaD mutant impaired in secretion of IpaB and IpaC and ipaB or ipaC mutants required for the formation of the translocon inserted in the host membrane. Rescue of ipaB and ipaC mutants with plasmids expressing IpaB or IpaC, respectively, restored the recruitment of G3CS to infected cells (Fig. 5B). Thus, these results show that acquisition the G3CS was dependent upon having a functional TTSS along with its translocon. Furthermore, our data suggest that it was rather the activity of the entire TTSS apparatus rather than a single effector triggering for the formation of G3CS.

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in Fig. 6A, mutant ipaC/ipaC351 recruited G3CS following internalization of the bacteria (arrows), indicating that TTSS-induced vacuole lysis was necessary and sufficient for the process of recruitment. Note that G3CS could not be seen with parental ipaC null mutant (Fig. 5B). In order to confirm the requirement of vacuole lysis for the formation of the G3CS, we actively induced vacuole lysis, following passive internalization by using an ipaC null mutant that was complemented by IpaC expressed from a plasmid containing an IPTG-inducible lac promoter (pHI3) (Page et al., 1999). The experimental procedure is depicted on Fig. 6B. In this experiment J774 macrophages were infected with mutant strain ipaC/pHI3. Following 20 min of bacterial infection, extracellular bacteria were carefully washed and infected cells were incubated with IPTG to induce the lac promoter, leading to intravacuolar induction of IpaC expression and functional assembly of the TTSS translocon. As can be seen in Fig. 6B, cells grown without IPTG did not display any G3CS in proximity to internalized bacteria similarly to ipaC null mutant (Fig. 5B), whereas cells grown with IPTG showed bacteria recruiting G3CS (arrows). These data confirm that the formation of G3CS was concomitant with the process of vacuole lysis.

Both Listeria and Salmonella sifA mutant recruit G3CS

We were interested in finding out whether other intracellular bacterial pathogens, both Gram-positive and -negative could recruit galectin-3 structures. We found that Listeria, which also lyse the phagocytic vacuole, induced formation of G3CS in J774 macrophages (Fig. 7A). Moreover, a hly mutant of Listeria monocytogenes, unable to lyse the phagocytic vacuole (Gedde et al., 2000), did not induce recruitment of G3CS (Fig. 7A). This result, combined with the results shown in Fig. 6, demonstrates that lysis of the vacuole with either a Gram-positive or a Gram-negative bacterium was sufficient for recruitment of G3CS in infected cells.

Unlike Listeria and Shigella, Salmonella bacteria that remain in a phagocytic vacuole used as a replicative niche (Garcia-Del Portillo et al., 2008). However, a sifA mutant is able to lyse the phagocytic vacuole and to replicate in
Fig. 6. G3CS appear at the step of vacuole lysis. Fluorescence microscopy of J774 macrophages infected by *S. flexneri* M90T mutants inducing or not the activity of TTSS. A. *ipaCipaC351 S. flexneri* strain (Barzu et al., 1997) was used to infect J774 macrophages for 20 min. B. Upper panel, a cartoon describing the different stages of the experiment. Lower panel, immunofluorescence images of the experiment. *ipaC*pHI3 *S. flexneri* strain (Page et al., 1999) was used to infect J774 macrophages for 20 min followed by addition of IPTG (200 μM) and gentamicin (50 μg ml⁻¹) for 1 h at 37°C, to induce IpaC expression.

Fig. 7. G3CS appear at the step of vacuole lysis of *listeria* and *salmonella* sifA. A. *Listeria monocytogenes* wild-type and *Δhly* were used to infect J774 macrophages for 20 min. B. *Salmonella* wild-type 12023 and sifA mutant were used to infect HeLa cells for 6 h. Arrows mark G3CS. Scale bar = 4 μm.
the cytosol (Beuzon et al., 2000; 2002). As shown in Fig. 7B, sifA mutant was able to recruit G3CS, while Salmonella wild-type bacteria was not. A small amount of intracellular Salmonella bacteria (about 5%) recruiting galectin-3 structures could be explained by the ability of a small amount of bacteria to lyse the vacuole (Beuzon et al., 2000; Brumell et al., 2002). Therefore, it is likely that the capacity to lyse the vacuole rather than the nature of the pathogen dictates the formation of G3CS.

Discussion

In this manuscript we show that galectin-3 accumulates in the vicinity of invading bacteria, both Gram-positive and Gram-negative, that lyse their phagocytic vacuole, but not in the vicinity of bacteria that do not lyse their phagocytic vacuole. The G3CS appeared in epithelial and macrophages cell lines and also in primary cells. More than half of intracellular Shigella (65%) were G3CS-positive according to our immuno-electron microscopy analysis in J774 macrophages. Our EM data showed that the G3CS localized to the membranes deriving from the phagosome and to a network of tubules and vesicles intersecting with the endocytic pathway. The direct localization of galectin-3 within the endocytic pathway has not been shown before. However, several previous studies correlated galectin-3 with the endocytic pathway. It has been previously shown in a proteomic analysis that galectin-3 was a part of phagosomal proteins (Garin et al., 2001). Also, proteomic mapping of exosomes revealed that galectin-3 was one of the proteins appearing in these vesicles of endosomal origin (Thery et al., 2001). Last, galectin-3 was shown to interact with the two lysosomal/late endosomal-lysosomal proteins, Lamp1 and Lamp2 (Dong and Hughes, 1997; Sarafian et al., 1998).

Galectin-3 probably does not bind Shigella directly. First, galectin-3 did not bind Shigella in vitro (data not shown). Second, our EM data suggest that galectin-3 was localized in the phagosome and in tubules and vesicles fused with it and not in close vicinity to the bacteria (Fig. 3). In addition, we showed that galectin-3 was recruited both by Gram-positive and Gram-negative bacteria. If galectin-3 indeed bound to bacteria, the interaction might have been with two different molecules in two types of pathogens. It seems to us less likely that this was the case and we favour an explanation in which galectin-3 was recruited to the phagosome without the need for direct interaction with the bacteria. Further experiments are required to study the manner of galectin-3 recruitment to the phagosome, either from the cytosol, in a similar manner to its recruitment to the plasma membrane in K-Ras4B signalling (Elad-Sfadia et al., 2004; Ashery et al., 2006) or from exocytosis of endomembranes to the phagocytic cup (Lee et al., 2007).

The G3CS appearing after bacteria internalization were visualized by galectin-3 labelling, yet we suspect that other molecule/s also participated in forming them. Our results show that LacNAc containing host N-linked glycans were likely to be required for galectin-3 incorporation within the structures. When we used an R186S galectin-3 mutant that had no LacNAc-binding activity, no G3CS could be seen following infection by Shigella. Moreover, CHO-Lec1 cell line, deficient in LacNAc-addition to N-linked glycans also showed no G3CS following infection (Fig. 4), whereas wild-type CHO cells and Lec2 cells deficient in sialylation did (data not shown). These results also correlate with the ability of the wild-type and Lec2, but not Lec1 CHO cells to bind externally added galectin-3, reflecting their glycan repertoire (Patnaik et al., 2006). Consequently, it seems reasonable that the G3CS contained a complex of at least galectin-3 and a host N-glycosylated protein. We should stress that the fact we could not identify G3CS by using gSL3 labelling in certain experiments cannot lead to the conclusion that corresponding structures did not exist. The formation, or not, of structures should be tested after finding at least one additional molecule labelling the structures and this remains to be further studied.

Nature of G3CS

It has been proposed that upon binding to oligosaccharides, the non-lectin domains of galectin-3 molecules may interact with one another, leading to their oligomerization. These oligomerized galectin-3 molecules are known as clusters or lattices that cross-link ligands on the cell surface (Massa et al., 1993; Ahmad et al., 2004). G3CS may be, in fact, such intracellular multivalent clusters of galectin-3 molecules interacting within themselves and with other protein/s yet to be identified. G3CS are likely to be lattices as they gradually grow and gain more galectin-3 labelling (Figs 2 and 3). The formation of these intracellular lattices may be triggered by the binding of N-glycan/s that is/are required to the formation of G3CS (Fig. 4). What may be the source of these N-glycan/s? We propose that these are cell-surface N-linked glycans that are exposed to the cytosolic compartment due to phagosome lysis, which is essential to the formation of G3CS (Figs 6 and 7). Binding of these cell-surface N-linked glycans to galectin-3 may trigger the formation of clusters of G3CS in a similar manner to extracellular clusters (Nieminen et al., 2007). Extracellular and plasma membrane galectin-3-containing lattices are functionally important for the turnover of cell surface receptors and the regulation of signalling in unique membrane microdomains (Furtak et al., 2001; Elad-Sfadia et al., 2004; Partridge et al., 2004; Chen et al., 2007; Nieminen et al., 2007). Clustered membrane microdomains resulting from
the binding of galectin lattices to certain glycoproteins serve as a preferential location of signalling (Pace et al., 1999; Brewer et al., 2002). In a similar manner, lattices of G3CS within the phagosome may serve as a platform for assembly of other proteins and the occurrence of signalling. Indeed, Dupont et al. (2009) could observe that following infection, the lysed membranes labelled by galectin-3 underwent polyubiquitination and then activation of the inflammasome, stress response and cell death signalling within the phagosome. Further studies are required to verify possible roles or a possible role of galectin-3 in such signalling.

**Galectin-3 as a tool to spot vacuole lysis**

What was clear from our observations with bacteria was that disruption of the vacuole by the TTSS in the case of *Shigella*, by haemolysin in the case of *Listeria* and by the absence of *sifA* protein of *Salmonella* was essential for the visualization of G3CS (Fig. 7). Moreover, the appearance of the structures could be induced upon activation of the TTSS resulting in vacuole lysis (Fig. 6B). While galectin-3 by itself was not required for vacuole lysis (data not shown), the G3CS were recruited only when there was a lysis process. When the vacuole was not lysed, both by *Shigella* and *Listeria* mutants, there was no G3CS recruitment. Furthermore, most *Salmonella* bacteria, which do not lyse the vacuole, did not recruit G3CS. We thus conclude that when G3CS were present, there was a certain extent of vacuole lysis. Our EM experiments cannot prove whether or not a vacuole was lysed and additional experiments are required to verify the exact timing of G3CS appearance during the process of the vacuole lysis. G3CS could be seen as early as about 5 min after *Shigella* infection of J774 macrophages. We do not know what was the extent of vacuole lysis at this time point and this question was further studied by using a novel assay to spot vacuole lysis. The results indeed showed that *Shigella* lysed the vacuole in less than 10 min and that G3CS are recruited to ruptured vacuoles fast after the beginning of the lysis, which points to the rapid dynamics of the process (K. Ray, et al., accepted). Our finding that 65% of the bacteria recruit G3CS means that these bacteria are most likely free in the cytoplasm. The other 35% may be in the process of vacuole lysis, not yet recruiting G3CS or that for some reason may not have lysed the vacuole. This last hypothesis can be further studied using the galectin-3 labelling and the novel assay for vacuole lysis proposed by K. Ray et al. (accepted).

If galectin-3 was indeed recruited upon vacuole lysis, then how could one explain its recruitment to phagosomes containing *Mycobacterium* (Beatty et al., 2002)? Indeed, this study showed binding to *Mycobacterium* phosphatidylinositolmannosides (PIMs) *in vitro*, yet this interaction was not demonstrated *in vivo* within the phagosome. *Mycobacterium* species are known to reside in the phagosome, yet there are reports of *Mycobacterium marinum*, motile in the cytoplasm (Stamm et al., 2003), of *Mycobacterium tuberculosis* free in the cytoplasm (McDonough et al., 1993) or performing cell-to-cell spread in tissue culture (Byrd et al., 1998). Consequently, it might be conceivable that galectin-3 was recruited to disrupted or partially disrupted vacuoles also in the case of *Mycobacterium* species.

Vacuole lysis is a process not yet fully understood, as compared with other stages in bacteria life cycle, such as invasion. This is partly due to the lack of markers associated with a disrupted, but not intact, phagosome. Currently, the methods for identifying vacuole rupture include EM analysis, usage of antibiotic markers and in the case of motile bacteria, such as *Shigella* and *Listeria*, the appearance of actin comet tail (Sansonetti et al., 1986; Goun et al., 1999; Paetzold et al., 2007). This actin tail is associated with bacteria that lysed the vacuole and are free in the cytoplasm. Both the usage of EM studies and antibiotics incorporation cannot be used in high-throughput studies. The appearance of actin tails is a late phenomenon, observed long after the vacuole had been lysed. There is a need for a reliable marker associated with the immediate process of vacuole lysis. We propose that galectin-3 can serve as such a marker. Examples for high-throughput assays may be screening mutants unable to lyse the vacuole with the purpose of identifying bacterial and host molecules essential for this process as well as studying host signalling before and after vacuole lysis. To our knowledge, this is the first cellular marker that can be used for these kinds of studies.

**Experimental procedures**

**Bacterial strains, cell lines, plasmids and reagents**

MDCK, MDCK-RCA' (kindly provided by Dr K. Simons, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Invitrogen). 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen). Caco-2 cells were grown in DMEM containing 10% fetal calf serum (invitrogen), 1% non-essential amino acids (Invitrogen), 100 µl⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen). J774 cells were grown in RPMI-1640-Glutamax supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen). J774 cells were grown in RPMI-1640-Glutamax supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen). CHO and CHO Lec1 cells were grown in RPMI-1640-Glutamax (invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen). SKBR3 cells were grown in RPMI-1640-Glutamax supplemented with 10% fetal calf serum, Sodium pyruvate 1% (PAA), non-essential amino acids 1% (PAA),

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100 U ml\(^{-1}\) penicillin and 100 µg ml\(^{-1}\) streptomycin. SKBR3 cells complemented with GFP-galectin-3 were grown in the same media supplemented with G418 (1 mg ml\(^{-1}\)) (Gibco, Invitrogen 10131-027). For transfection of SKBR3 cells, the cells were seeded in 24-well plates at a density of 50,000 cells per well in antibiotic-free medium. After 24 h culturing of the SKBR3 cells were transfected using the Lipofectamine 2000 (Invitrogen) according to manufacturer’s description and the pEGFP-C1 plasmid containing either galectin-3 or galectin-3R186S (Delacour et al., 2007) using 0.8 µg DNA per well. After 24 h the medium of the transfected cells was switched to antibiotics containing medium. Selection of stably transfected cells were performed using 1 mg ml\(^{-1}\) of G418 in cell culture media.

All cells were grown at 37°C, in an incubator supplemented with 10% CO\(_2\). The \(S.\ flexneri\) serotype 5a strain M90T was used as a wild-type strain (Sansonetti et al., 1982). The non-invasive, plasmid-cured, BS176 was described previously (Sansonetti et al., 1982). The ipaC- and ipaB-mutants were described previously (Menard et al., 1993). IpaC351 was described previously (Barzu et al., 1997) and ipaC\(^{+}\)H3 was described in Page et al. (1999). \(L.\ monocytogenes\) was previously described in Mounier et al. (1990). \(Salmonella\) wild-type 12023 and sifA mutant were previously described in Sansonetti et al., 1986; Beuzon et al., 2002). M90T-GFP is the \(S.\ flexneri\) M90T strain transformed with pEGFP (Clontech).

**Infection experiments**

Infection of cells by \(Shigella\) was performed as described (Adam et al., 1996). Briefly, 16 h before the experiment, cells were plated at a density of 2–5×10\(^5\) cells per well onto a 12-well plate containing 14 mm glass coverslips. Bacteria were grown in TCS (Difco) overnight until mid-exponential phase, centrifuged for 5 min, washed and resuspended in DMEM or RPMI. Bacteria were added to cells with a multiplicity of infection (moi) of 100–150 bacteria per cell. To synchronize infection, cells and bacteria were centrifuged at 700 g for 10 min at 25°C, using a Rotixa/RP swinging-bucket centrifuge equipped with tray holders (Hettich). Samples were incubated for 20–30 min at 37°C to allow bacterial invasion and then washed with PBS. For prolonged infections (over 30 min), infected cells were incubated with gentamicin (50 µg ml\(^{-1}\)) at 37°C. \(Listeria\) infection was performed in a similar manner as described previously (Mengaud et al., 1996).

\(Salmonella\) wild-type 12 023 and sifA mutant were diluted at 1/30 from overnight culture in new broth TCS for 3 h 30 min with agitation. Infection was performed at moi 100/1 for 15 min in CO\(_2\) 37°C, washed once with DMEM then incubate in DMEM 10% fetal calf serum and gentamicine 100 µg ml\(^{-1}\) for 1 h. Cells were washed once more and incubated for 5 h in DMEM 10% fetal calf serum and gentamicine 16 µg ml\(^{-1}\).

**Immunofluorescence analysis**

Infected cells on glass coverslips were fixed with 3.7% paraformaldehyde in PBS for 30 min, washed in PBS and neutralized with 0.1 M glycine in PBS for 5 min. The cells were then permeabilized with 0.5% (v/v) Triton X-100 in PBS for 2 min and saturated in 0.2% gelatin for 5 min. The cells were then incubated with specific mono- or polyclonal antibodies diluted in PBS. Galectin-3 was immunostained using a specific monoclonal anti-galectin-3 M3/38 antibody used at 1/100 dilution (kindly given by Dr. M. Benhamou, INSERM U699, Faculté de Médecine Xavier Bichat, Paris) or Mouse monoclonal anti-Galectin-3 antibody (BD Transduction 556904) used at 1/100 dilution. Bacteria were immunostained using anti-rabbit polyclonal \(S.\ flexneri\) LPS antibody (A. Phalipon, Institute Pasteur, Paris) used at 1/200 dilution. \(Salmonella\) serum anti-rabbit AgO at 1/300.

**Actin distribution analysis** was performed by FITC-conjugated phaloidin (Sigma), used at 1/500 dilution. Secondary Abs used were anti-rat IgG antibody conjugated to fluorescein FITC (Jackson Immunoresearch 712-095-150), anti-rabbit IgG Ab conjugated to rhodamine (TRITC) (Jackson Immunoresearch 111-025-144), both used at 1/200 dilution, GAM IgG-Alexa555, and GAR-Marina blue (Molecular Probes) both used at 1/500 dilution. Phalloidin-FITC was from Sigma. TUNEL staining was performed with ‘in situ cell death detection kit, POD (Roche, cat N° 11 684 817 910) according to the manufacturer’s instructions. Coverslips were mounted with DABCO (1,4-Diazabicyclo-[2.2.2] octane Sigma D-2522). Preparations were either observed by a conventional fluorescence microscope (BH2 RFCA, Olympus Optical Co. Ltd) or with an AxioImager microscope (Zeiss) fitted with AxioCam MRm camera. Preparations were also observed by a confocal laser scanning microscope (Zeiss Axiopt), Images were acquired and assembled using Metamorph and Adobe Photoshop softwares.

**EM experiments**

A total of 2.5×10\(^5\) \(J774\) cells were grown overnight in 10 cm plates. A coverslip was always included to verify efficiency of infection by IF in every experiment. Infection was performed in a similar manner to infection followed by IF. Cells were fixed either 4% PFA or with 2% PFA and 0.1% glutaraldehyde FA in 0.1 M phosphate buffer pH 7.4 for 3 h at room temperature. Processing of cells for ultrathin cryosectioning and immunolabelling was done as described previously (Slot et al., 1991). Species specific secondary antibodies conjugated to gold were purchased from British Biocell (Cardiff, UK). For BSA-gold experiments, BSA-gold conjugated to 5 nm colloidal gold (UMC Utrecht, the Netherlands) was used at a final absorbance of 5 at 520 nm. \(J774\) macrophages were incubated for 2 h at 37°C in RPMI-1640-GLutamax without FCS. A volume of 170 µl of BSA-gold was added to 5 ml medium and cells were incubated for 30 min at 37°C. Following incubation, cells were washed 3 times with warm medium (RPMI-1640-GLutamax) and incubated with medium containing 1% BSA for 10 min. Infection was performed in a similar manner to infection followed by IF. Cells were then fixed in the same manner described above.

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