Citrobacter rodentium Infection Induces MyD88-Dependent Formation of Ubiquitinated Protein Aggregates in the Intestinal Epithelium

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Abstract

\textit{Citrobacter rodentium} utilizes a type 3 secretion system (T3SS) to inject effector proteins into host intestinal epithelial cells, causing structural and functional changes in these cells during infection. Here, we examined the effects of \textit{C. rodentium} infection on host cell protein ubiquitination in vivo. We observed the appearance of ubiquitinated protein (Ub\textsuperscript{+}) aggregates in intestinal epithelial cells near the site of bacterial attachment. Formation of aggregates was dependent on T3SS activity and the effector translocated intimin receptor (Tir). Aggregates formed at 6 days after infection, when bacterial loads were maximal, but were absent at 12 days. Aggregates were not observed in MyD88\textsuperscript{-/-} mice. Aggregate formation correlated with MyD88-dependent induction of NADPH oxidase 1, implicating reactive oxygen species in their formation. Aggregates were also observed in gastric tissues of mice infected with \textit{Helicobacter pylori}. This is the first report describing the formation of Ub\textsuperscript{+} aggregates in vivo during enteric infection, and reveals that this phenotype is dependent on both bacterial and host factors. Our experiments extend previous in vitro studies suggesting that Ub\textsuperscript{+} aggregates play an important role in the initiation of immune responses to infection. Ub\textsuperscript{+} aggregates are a novel marker of the cellular response to enteric pathogens and will be useful for studies of host-pathogen interactions in vivo.

Introduction

The mucosal pathogen, \textit{Citrobacter rodentium} belongs to a family of enteric pathogens, including enteropathogenic \textit{Escherichia coli} and enterohemorrhagic \textit{E. coli}, that utilize attaching and effacing (A/E) lesions to colonize the gastrointestinal tract [1]. Intestinal A/E lesions are defined as a localized destruction of the brush-border microvilli caused by the intimate attachment of the pathogen to the plasma membrane of the intestinal epithelial cells [for review, see 1, 2]. \textit{C. rodentium} is a mouse pathogen, so it is often used as an in vivo model for A/E lesion-forming pathogens such as enterohemorrhagic \textit{E. coli}.

A bacterial type III secretion system (T3SS) genetically encoded on the locus of enterocyte effacement pathogenicity island is essential for the formation of A/E lesions [3, 4]. \textit{C. rodentium} uses its T3SS to translocate effector proteins such as the translocated intimin receptor...
(Tir), mitochondrial associated protein (Map), EspF, EspG, and EspH into host cells [5]. Tir is essential for pathogenesis [6, 7], whereas Map contributes to injury of the intestinal mucosa [8]. In fact, a Δmap strain of C. rodentium is attenuated in its ability to colonize mice [9, 10]. The EspF homolog in enteropathogenic E. coli has been shown to induce host cell death [11]. Through these and other effector proteins, C. rodentium establishes infection in the gut, leading to many structural and functional changes within the intestinal epithelium.

C. rodentium infection causes thickening of the colonic mucosa (colonic hyperplasia), goblet cell depletion and a local Th1 and Th17 immune response [1, 12, 13]. In addition, C. rodentium can also severely compromise colonic epithelial barrier function [8, 14]. The colonization of C. rodentium in normal adult mice peaks at 6–8 days when intestinal bacterial counts are maximal and inflammation is evident [1, 15–17]. C. rodentium infection is transient and typically resolves by 28 days [13]. Clearance of C. rodentium from the host starts at approximately 14 days after infection and adaptive immunity takes an active role in clearance [13]. Since C. rodentium infection is cleared naturally by the host, this makes it an excellent model for studying successful innate and adaptive immune mechanisms at the intestinal mucosa.

Epithelial cells at intestinal mucosal surfaces comprise the first line of defense against microbial pathogens [18, 19]. Toll-like receptors (TLRs) detect conserved pathogen-associated molecular patterns (PAMPs) and play a crucial role in triggering immune responses to infection [20]. Intestinal mucosal tissues are the major sites of PAMP-TLR signaling because of their close proximity to luminal bacterial and food antigens [21]. It is known that during initial infection, C. rodentium PAMPs activate both TLR4 and TLR2 and potentially other TLRs [22, 23]. Engaged TLRs (with the exception of TLR3) trigger signaling through the adaptor molecule myeloid differentiation factor 88 (MyD88) [24, 25]. MyD88-dependent responses include nuclear factor κB activation, which plays a central role in pro-inflammatory cytokine production [18]. A recent report indicates MyD88-dependent signaling within C. rodentium-infected epithelial cells plays an important role in both inflammatory responses and maintenance of mucosal homeostasis [26].

Epithelial cells lining the colon play an active role during infection in vivo, not only acting as a physical barrier, separating luminal bacteria and immune cells, but also secreting cytokines and antimicrobial peptides that contribute to both host defense and tissue damage. Epithelial cells also express the inducible form of nitric oxide synthase (iNOS) in response to infection. iNOS is capable of producing large quantities of reactive nitrogen intermediates and nitric oxide to exert an antimicrobial response. iNOS expression by epithelial cells in the gut contributes significantly to C. rodentium clearance [27]. Another integral part of the epithelial host innate immune response to pathogens is the generation of reactive oxygen species (ROS) via members of the NADPH oxidase family, NADPH oxidase 1 (NOX1) and dual oxidase 2 (Duox2) which are expressed in the gastrointestinal tract [28]. Interferon-γ (IFN-γ), a pro-inflammatory cytokine secreted by T cells and natural killer cells in response to intestinal infection, can upregulate NOX1 expression in epithelial cells of the large intestine [29, 30]. There are also data supporting a role for Duox2 activation in limiting infection at the mucosal epithelia [31]. These data suggest that NADPH oxidases may constitute an early host defense response by epithelial cells against pathogens, although the exact mechanisms are unclear.

Cellular responses to infection include the formation of ubiquitinated protein (Ub⁺) aggregates [32, 33]. The accumulation of cytosolic polyubiquitinated protein aggregates was first characterized in dendritic cells exposed to lipopolysaccharide (LPS) [34] and was subsequently observed in macrophages in response to various PAMPs [32]. The Ub⁺ aggregates are thought to comprise mainly defective ribosomal products (DRPs) that are normally degraded by the proteasome [34], a principal source of peptides for MHC class I antigens [35, 36]. Aggregate formation may occur as a result of oxidative stress [37, 38] and/or translational upregulation associated with infection [39]. The Ub⁺ aggregates (also referred to as dendritic cell aggresome-like induced structures or DALIS) are hypothesized to act as antigen storage compartments for host and pathogen-derived MHC class I restricted antigens [33, 34]. Indeed, Listeriolysin O, a major antigen required for the clearance of Listeria monocytogenes infection has been observed to localize to Ub⁺ aggregates [40]. Therefore, Ub⁺ aggregate formation appears to be an important cellular response to infection. In support of this notion, the intracellular pathogen Legionella pneumophila was recently shown to disrupt Ub⁺ aggregate formation [41]. Ub⁺ aggregates have so far been studied exclusively in leukocytes in vitro. Whether these aggregates are formed in response to bacterial infection in vivo and whether other cell types display Ub⁺ aggregate formation during infection is presently unknown.

In the present study, we examined subcellular protein ubiquitination events in colonic tissues of mice infected with the murine enteric pathogen C. rodentium. We show...
that Ub+ aggregates form in the cytosol of colonic epithelial cells during infection and that these structures are transient in nature, and associated with acute inflammatory responses. Furthermore, we examined the pathogen- and host-driven factors that are responsible for Ub+ aggregate formation. Our observations indicate that the appearance of Ub+ aggregates in the epithelial cells surrounding the site of bacterial attachment in vivo is a MyD88-dependent event and a novel marker of infection. The formation of these structures may contribute to an inflammatory or cellular response to infection.

Material and Methods

Animals
C. rodentium-infected mice: 6- to 8-week-old C57BL/6 mice were obtained from Charles River. MyD88−/− mice were raised in our animal facility at the Child and Family Research Institute in sterilized, filter-topped cages, handled in tissue culture hoods, and fed autoclaved food and water under specific pathogen-free conditions. Sentinel animals were routinely tested for common pathogens. Animals were obtained from Jackson Laboratories. All mice had free access to fluids and chow. Mice were challenged with H. pylori strain SS1 (10⁸ CFU in 100 μl) orogastrically using a 22-G gastric gavage needle 3 times in a 5-day period. Mice were sacrificed after 2 weeks by intraperitoneal injection of 0.1 ml chloral hydrate. Animal treatment and care was carried out according to the guidelines outlined by the Laboratory Animal Services at the Hospital for Sick Children, Toronto.

Tissue Removal and Fixation
Colon tissues were collected as described previously [23], in 10% neutral buffered formalin (Fisher) for histological analyses. The stomach was divided into longitudinal sections, each section containing the squamous, body and antrum portions of the stomach. Separate segments of stomach were immediately placed in 10% neutral buffered formalin and later embedded in paraffin for histological analysis.

Cell Lines
Henle-407 human epithelial cell line (American Type Culture Collection) was maintained in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10% FBS (Wisent) at 37°C under a 5% CO₂ and 95% air atmosphere without antibiotics.

siRNA Treatment
siGenome NOX1 siRNA (Smartpool, M-010193-01; Dharmacon), NOX4 siRNA (Smartpool, M-010194-01; Dharmacon), Cyba (p22) siRNA (Smartpool, M-011020-01-005; Dharmacon) and Standard siControl Nontargeting siRNA pool (D-001206-13-20; Dharmacon) were used at a final concentration of 100 nM for 48 h before bacterial culture filtrate (BCF) stimulation.

BCF Generation and Stimulation
BCF were generated as described by Raju and Jones [42]. Growth media with or without 20% BCF was added to cells and incubated for 12 h before fixation on coverslips with 2.5% PFA.

Western Blotting
Confirmation of knockdown was assessed via Western blotting. Samples were separated on SDS/12% PAGE gels, transferred to polyvinylidene difluoride membranes, blocked overnight in 5% milk solids, and probed with antibodies: rabbit anti-NOX1 (Abcam), rabbit anti-p22 (generous gift from Dr. Mark T. Quinn) or mouse anti-β-tubulin (Sigma).

Immunofluorescence and Microscopy
The paraffin-embedded intestine and stomach sections were mounted onto slides and dried for staining. Glass-mounted sections were cleared from paraffin with xylene and rehydrated by sequential washings with graded ethanol solutions (95–70%). From here, slides were subjected to permeabilization [phosphate-buffered saline, PBS−/−, (1 x DPBS/modified + calcium + magnesium; HyClone Thermo Scientific) with 0.1% Triton X-100] for 15 min at room temperature and sections were incubated for 3 h in blocking buffer [10% normal goat serum (Wiset) and 0.2% saponin (Calbiochem) in PBS−/−]. Sections were then incubated overnight at 4°C with the primary antibodies. The slides were washed with PBS, followed by the specific secondary antibodies for 2 h. The following antibodies were used for immunofluorescence: (1) mouse monoclonal antibody (mAb) FK2 (Biomol; 1:100), (2) rabbit polyclonal against C. rodentium (generously provided by Dr. Philip Sherman, Hospital for Sick Children; 1:100), (3) rabbit polyclonal [42] against H. pylori (Dako; 1:100), (4) rabbit polyclonal against iNOS (Santa Cruz; 1:100) and (5) goat polyclonal against NOX1 (MOX) (Santa Cruz; 1:100). In order to visualize NOX1 staining in vivo, tissue sections were subjected to an antigen retrieval treatment. Briefly, after sections are deparaffinized and rehydrated using graded ethanol baths, the sections were boiled in antigen retrieval buffer consisting of 10 mM Tri-sodium citrate dihydrate, 0.05% Tween 20 at pH 6.0. The boiling was done in a microwave for 30 min. After approximately 30 min of cooling, the sections were then washed in PBS and incubated in blocking buffer. Secondary antibodies used were: (1) Alexa 488-conjugated goat anti-mouse IgG, (2) Alexa 568-conjugated goat anti-rabbit IgG and (3) Alexa 488-conjugated donkey anti-goat (Molecular Probes). The nuclei were stained using DAPI for 30 min to facilitate cell parameters.

In vitro immunofluorescence staining was carried out as previously described [38]. Primary antibody was used was mouse mAb FK2 (Biomol; 1:100), along with DAPI nuclear stain. Secondary antibody used was goat anti-mouse Alexa conjugate. For determining the presence of intracellular bacteria, immunostaining of extracellular bacteria before 0.1% saponin (Sigma) permeabilization was used.

Tissue samples were analyzed using a Leica DMIRE2 fluorescence microscope (63× and 100× objective) and OpenLab software. In vitro images were confocal z slices taken by a Quorum
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spinning disk microscope (Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu back-thinned electron multiplying charge-coupled device camera, spinning disc head and Volocity 5 software). Mirax images were done at the Imaging Facility at the Hospital for Sick Children in Toronto. A Zeiss Mirax Scanner was used to obtain images using a 20×/0.80 Zeiss objective in combination with a Marlin F146-C. To quantitate Ub+ aggregates in tissue sections, Alexa 568 intensity measurements (for the FK2 mAb) were done using Volocity Imaging software. Intensity was measured by determining the fluorescence signal (pixels) divided by the area. Background intensities were subtracted from the calculated amounts. All images were imported into Adobe Photoshop and assembled in Adobe Illustrator.

**Results**

**Ub+ Aggregates Are Observed in Colonic Epithelial Cells during *C. rodentium* Infection of Mice**

*C. rodentium* bacteria induce colonic epithelial hyperplasia and mucosal inflammation in mice [1]. To assess whether Ub+ aggregates form during in vivo bacterial infection, we examined colonic tissue sections from *C. rodentium*-infected mice. A mAb (FK2) that recognizes both mono- and polyubiquitinated proteins, but not free ubiquitin, was utilized for our studies [43]. Immunohistochemical analysis revealed large cytosolic ubiquitinated aggregates in intestinal tissues infected with *C. rodentium* (fig. 1a, arrows). These structures were not evident in uninfected mice.

To determine if Ub+ aggregates correlated with bacterial infection, we utilized an immunofluorescence approach. Sections from intestinal tissue were prepared from C57BL/6 mice infected with *C. rodentium* for 6, 8 and 12 days. Tissue sections were co-stained with the FK2 mAb and a rabbit anti-*Citrobacter* antibody to observe bacterial location and analyzed by epifluorescence. All intestinal tissues contained a small number of infiltrating immune cells that stained nonspecifically with secondary antibodies (fig. 1b, arrowheads). We also observed cytoplasmic Ub+ aggregates in the 6-day infected intestine but not in the uninfected controls (fig. 1b). Closer analysis revealed that cytoplasmic Ub+ aggregates were present in epithelial cells in highest abundance near the site of bacterial contact with the epithelium. A small number of aggregates were found in 8-day infected tissues when the...
Infection is typically starting to resolve (fig. 1b). Interestingly, we observed a diffuse ubiquitinated protein staining and the disappearance of Ub+ aggregates in the 12-day infected intestine (fig. 1b), much like the uninfected tissue.

In order to quantify Ub+ aggregates in these samples, we used a Mirax scanner to determine the intensity of the FK2 antibody signal (fig. 1c–e; see Material and Methods). Using 3 independent experiments per condition, the intensity of the Alexa 568 (red) signal was determined in tissues. Since our immunofluorescence data indicated that Ub+ aggregates were near sites of bacterial attachment, we subdivided our analysis into two regions of interest: one region closest to luminal surface, where bacterial contact sites are located, that is <50 μm, and a second region distal to the bacterial contact site (>50 μm). As expected, we observed the highest intensity for Ub+ aggregates in tissue from 6-day infected mice in the region <50 μm from the infected luminal edge (fig. 1d, f). Note that this signal was significantly different from the region >50 μm in the same tissue. This signal was not present in tissue from 12-day infected animals (fig. 1e, f). As a control, we examined tissues from 6-day infected mice stained with the secondary antibody alone, and observed a low signal that was significantly different from tissues stained with both primary and secondary antibody (fig. 1c, f). These findings demonstrate that Ub+ aggregates are found in intestinal epithelial cells that are proximal to C. rodentium infection and suggest that this cellular phenotype may be involved in the host immune response to acute infection.

We also examined another enteric infection model, H. pylori-infected murine gastric tissue. Examination of H. pylori-infected mucosa by immunohistochemistry revealed cytoplasmic Ub+ aggregates in the gastric epithelium surrounding the lumen of the gastric glands, an area where H. pylori is known to reside (fig. 2a, arrows). These structures were not evident in uninfected mice. An immunofluorescence approach was also taken to examine Ub+ aggregates in H. pylori-infected gastric tissue. Stomach sections from C57BL/6 mice infected for 2 weeks with H. pylori were co-stained using mAb FK2, to visualize Ub+ aggregates, and an anti-H. pylori antibody and DAPI to identify the nucleus. When examining infected stomach sections we observed Ub+ aggregates in gastric epithelial cells (fig. 2b, arrow) in contrast to a diffuse cytosolic ubiquitination signal in the uninfected control. These Ub+ aggregates appeared larger and in greater quantity in cells proximal to the site of bacterial colonization (fig. 2b).

Taken together, these observations demonstrate that Ub+ aggregate formation occurs as a result of enteric infection in both the stomach and large intestine. Furthermore, in the case of C. rodentium infection, we determined both microscopically and quantitatively that Ub+ aggregate formation correlates with bacterial contact with epithelial cells. We decided to further characterize Ub+ aggregate formation using C. rodentium infection in vivo.

Type III Secreted Effectors Are Required for Ub+ Aggregate Formation by C. rodentium

C. rodentium use type III secreted effectors to initiate A/E lesion formation. To investigate whether bacterial effectors could influence Ub+ aggregate formation in vivo, C57BL/6 mice were infected with wild-type and various effector mutants of C. rodentium and Ub+ aggregate formation was examined. Murine colon sections were stained for Citrobacter and Ub+ aggregates in mice infected with Δtir, Δmap, ΔespF, ΔespG and ΔespH C. rodentium for 6 days and compared with wild-type C. rodentium-infected mice. Mice infected with ΔespG or ΔespH mutants displayedUb+ aggregates similar to wild-type infection (fig. 3a, b), consistent with the fact that these effectors have little or no effect on C. rodentium pathogenesis in vivo [44, 45]. However, mice infected with ΔespF or Δmap mutants showed a marked decrease in the number and size of Ub+ aggregates compared to mice infected with wild-type bacteria (fig. 3a). Quantitative analysis indicated that tissues infected with a Δmap mutant had less Ub+ aggregate staining compared to tissues infected with wild-type bacteria (fig. 3b). This is consistent with previous studies demonstrating that a Δmap mutant has a significant impairment in its ability to competitively colonize mice compared to wild-type bacteria [8]. During infection, Map is thought to promote mitochondrial dysfunction and barrier disruption [8]. EspF is also critical for disrupting epithelial tight junctions, as assessed at 7 days after infection, thereby contributing to the diarrhea symptoms caused by A/E pathogens [46, 47]. The effector Tir plays a critical role in establishing A/E lesions and neither bacterial colonization nor colonic hyperplasia is detected in mice infected with Δtir C. rodentium [15]. As expected, we did not observe Ub+ aggregate formation in mice infected with a Δtir mutant (fig. 3a, b).

We conclude that T3SS effectors required for bacterial colonization of mice are required for Ub+ aggregate formation. It is also noteworthy that the level of virulence of C. rodentium attenuation (imported by specific T3SS effector mutations) correlates with diminution of the Ub+
Fig. 2. *H. pylori* induces the formation of Ub\(^+\) aggregates in gastric epithelial cells during infection of mice. **a** Immunohistochemistry staining of gastric tissue of C57BL/6 mice uninfected and infected by *H. pylori* strain SS1 (12 days after infection). Tissue sections were stained for mono- and polyubiquitin using the FK2 antibody (top panels) and hematoxylin and eosin (bottom panels). The tissue infected with *H. pylori* contains Ub\(^+\) aggregates (arrows), whereas the noninfected tissue is devoid of these aggregates. Note that in the bottom panel, aggregates are evident in the cells surrounding the lumen of the gastric glands, an area where *H. pylori* is known to reside. **b** Representative immunofluorescence micrographs of gastric epithelial cells of C57BL/6 mice infected with wild-type *H. pylori* for 14 days. Tissue was triple stained for Ub\(^+\) (red), using the mAb FK2, anti-*H. pylori* antibody (green) and a nuclear DAPI stain (blue). Ub\(^+\) aggregates (arrows) were detected in tissue infected with *H. pylori* compared to no aggregates in the uninfected tissue. Arrowheads show infiltrating immune cells that stained non-specifically with secondary antibodies. Scale bars = 10 μm. Asterisks indicate position of *H. pylori*.
Fig. 3. *C. rodentium* type III secreted factors influence the presence of Ub+ aggregates in infected intestinal epithelia. a Representative immunofluorescence micrographs of intestinal epithelial cells of C57BL/6 mice infected with wild-type *C. rodentium*, its isogenic Δtir mutant, Δmap mutant, ΔespF mutant and ΔespH mutant for 6 days after infection. Tissue was triple stained for Ub+ (red), using the mAb FK2, anti-Citrobacter antibody (green) and a nuclear DAPI stain (blue). Ub+ aggregates (arrows) were only detected in tissue infected with wild-type *C. rodentium* and the *C. rodentium* isogenic ΔespH mutant. Ub+ aggregates do not form in epithelial cells in mice infected with the *C. rodentium* Δtir mutant, Δmap mutant or ΔespF mutant. Scale bars = 10 µm. b Quantitative analysis of Mirax micrographic images, indicating the intensity measurements of the Alexa 568 (red) signal in 3 different intestinal tissue samples of C57BL/6 mice infected with wild-type and mutant *C. rodentium*. Intensity was measured by determining the fluorescence signal (pixels) divided by the area. Background intensities were subtracted from the calculated amounts. Intensity signals were calculated at <50 µm from the luminal edge and >50 µm from the luminal edge. A higher intensity signal was noticed in tissue <50 µm from the luminal edge infected with wild-type *C. rodentium* for 6 days compared to mice infected with Δmap and Δtir mutant *C. rodentium*. Results are the average (±SD) of 3 independent experiments. Asterisks indicate significant difference from the 6-day infected <50 µm count (* p < 0.1; ** p < 0.01; *** p < 0.00001), as determined by two-tailed, unpaired t test.
aggregate formation response. These data and the kinetics of Ub+ aggregate formation noted above imply that detection of bacterial loads in the intestinal lumen initiates the formation of these structures.

**MyD88 Is Required for Ub+ Aggregate Formation**

One mechanism by which bacteria are detected within the intestinal lumen is through recognition by TLR. The primary pathway through which TLR trigger inflammation against bacterial infections is via MyD88-dependent signaling [48–50]. Therefore, we chose to investigate whether loss of TLR/MyD88 signaling would have an impact on Ub+ aggregation in response to C. rodentium infection. C57BL/6 and MyD88−/− mice were infected with C. rodentium, and colonic sections were obtained 6 days after infection. Tissue sections were stained for Citrobacter and Ub+ aggregates. MyD88−/− mice infected with C. rodentium showed a diffuse staining for ubiquitinated proteins, with no evidence of Ub+ aggregates (fig. 4b). In contrast, C57BL/6 (control) mice infected with C. rodentium showed characteristic Ub+ aggregates in intestinal epithelial cells surrounding the infection (fig. 4a, arrows). Quantitative analysis showed that greater levels of Ub+ aggregate staining were present in the C57BL/6 (control) mice infected with C. rodentium compared to the MyD88−/− mice infected with C. rodentium (fig. 4c). These findings reveal that MyD88 plays an important role in signaling the formation of Ub+ aggregates during infection.

**Ub+ Aggregate Formation Precedes iNOS Expression in C. rodentium-Infected Mouse Colonic Epithelial Cells**

iNOS catalyzes the production of nitric oxide, a mediator important for innate immune defenses and signal transduction [51]. Epithelial cells strongly express iNOS during infection [52–54]. It has been shown previously that iNOS expression contributes to C. rodentium clearance [27]. Therefore, we examined whether iNOS expression correlates with Ub+ aggregate formation. C57BL/6 mice were infected with wild-type C. rodentium for 6 and 12 days. Colonic tissues were immunostained using an anti-iNOS antibody and FK2 mAb to visualize Ub+ aggregates. We found that iNOS expression did not appear until day 12 after infection, by which time Ub+ aggregates had already disappeared (fig. 5). iNOS-positive staining occurred in the epithelial cells surrounding the lumen of the infected colon, whereas in the uninfected control and 6-day infected tissues, iNOS staining was diffuse and Ub+ aggregates were evident (fig. 5). Colonic epithelial cells were found to be the major cellular source of iNOS during C. rodentium infection at 12 days, consistent with previous studies [27]. Therefore, we conclude that Ub+ aggregate formation does not correlate with iNOS expression, suggesting that nitric oxide production does not contribute to Ub+ aggregate formation during C. rodentium infection.

**NOX1 Expression in the Colon Is Induced in a MyD88-Dependent Manner and Correlates with Ub+ Aggregate Formation**

NOX1 is expressed in the colon [55–57]. Human epithelial cell lines were shown to exhibit high NOX1-mediated ROS production in response to flagellin from Salmonella [30]. Additionally, LPS from H. pylori was found to stimulate increased ROS production via NOX1 in human T84 cells [59]. Since the TLR-MyD88 signaling pathway leads to the production of IFN-γ and ROS is an important defense against bacterial infections, we decided to examine NOX1 expression in intestinal tissues infected with C. rodentium. We found NOX1 expression increased in C57BL/6 mice infected with C. rodentium compared to the MyD88−/− mice infected with C. rodentium (fig. 6a). This was particularly evident in epithelial cells surrounding the luminal site of infection. Indeed, Ub+ aggregates were visible in the epithelial cells surrounding the infection of the wild-type mice, but not the MyD88−/− mice (fig. 6b). We also looked at a wider section of tissue using Mirax scanning microscopy (×20 magnification). An increase in NOX1 expression was observed in infected mice in the cells proximal to the site of infection (fig. 6a). This increase in NOX1 expression was not observed in the MyD88−/− mice (fig. 6a). We conclude that NOX1 expression is upregulated in a MyD88-dependent manner during C. rodentium infection, an event that correlates with production of Ub+ aggregate formation.

**NOX1 Expression Is Required for Ub+ Aggregate Formation in Henle Cells after Stimulation with BCF**

Previously, we reported that cellular oxidative stress, along with other types of stresses, results in the formation of ubiquitinated aggregates [38]. Furthermore, we reported that ROS have been shown to contribute to Ub+ aggregate formation in a rat model of type II diabetes [37]. Thus, we hypothesized that the upregulation of NOX1 during C. rodentium infection may contribute to the formation of Ub+ aggregates. Recently, studies conducted by Necchi et
al. [60] indicated that *H. pylori* secreted factors cause the accumulation of polyubiquitinated proteins in the gastric epithelium, both in vitro and in vivo. Thus, we examined the contribution of NOX1 activity to Ub+ aggregate formation in the presence of *H. pylori* BCF. We observed that 12-hour BCF stimulation of Henle human intestinal epithelial cells resulted in a dramatic increase in Ub+ aggregate formation (fig. 7a, b). Furthermore, this increase was abolished when either p22, a critical subunit of NOX1–4, or NOX1 expression was reduced by siRNA silencing.

Fig. 4. MyD88 signaling induces the formation of Ub+ aggregates. Representative immunofluorescence micrographs of intestinal epithelial cells of mice infected with wild-type *C. rodentium* for 6 days after infection. Tissue was triple stained for Ub+ (red), using the mAb FK2, anti-Citrobacter antibody (green) and a nuclear DAPI stain (blue). a Ub+ aggregates (arrows) present in infected intestinal epithelial sections from C57BL/6 mice. b The absence of Ub+ aggregates present in infected intestinal epithelial sections from MyD88−/− mice. Scale bars = 10 μm. c Quantitative analysis of Mirax micrographic images, indicating the intensity measurements of the Alexa 568 (red) signal in 3 different intestinal tissue samples of C57BL/6 and MyD88-deficient mice infected with wild-type *C. rodentium*. Intensity was measured by determining the fluorescence signal (pixels) divided by the area. Background intensities were subtracted from the calculated amounts. Intensity signals were calculated at <50 μm from the luminal edge and >50 μm from the luminal edge. A higher intensity signal was noticed in C57BL/6 tissue <50 μm from the luminal edge infected with wild-type *C. rodentium* for 6 days compared to MyD88-deficient mice infected with wild-type *C. rodentium*. Results are the average (±SD) of 3 independent experiments. Asterisks indicate significant difference from C57BL/6 mice infected for 6-day infected <50 μm count (*p < 0.01), as determined by two-tailed, unpaired t test.
These results suggest a critical role for NOX1 expression in the formation of Ub⁺ aggregates in intestinal epithelial cells.

**Discussion**

To date, the formation of Ub⁺ aggregates has been studied exclusively in leukocytes in vitro [32, 34, 39, 61, 62]. Here, we report for the first time that bacterial infection can cause the formation of Ub⁺ aggregates in intestinal epithelial cells in vivo. The fact that these aggregates are formed in epithelial cells is novel and therefore demonstrates that this phenotype is not exclusive to leukocytes. The early presence of Ub⁺ aggregates at 6 days after infection suggests that formation of these structures plays an important role in early host immune responses. Ub⁺ aggregates studied in dendritic cells and macrophages in vitro have previously been described as transient in nature [38, 39]. Consistent with this, we observed the disappearance of Ub⁺ aggregates in vivo 12 days after infection. While this may reflect a transient response, it should also be noted that the turnover of epithelial cells in the colon would mean that the cells expressing these aggregates at day 6 would have been shed by day 12. Moreover, epithelial cell proliferation and turnover in the *C. rodentium*-infected colon is maximal at day 12, suggesting that the absence of the Ub⁺ aggregates at this time may reflect an inability for MyD88 signaling to induce their formation before they are sloughed into the colonic lumen.

Exposure of leukocytes to PAMPs is sufficient to initiate Ub⁺ aggregate formation in vitro [32, 34]. Our studies suggest that exposure of intestinal epithelial cells to bacterial PAMPs (through T3SS activity and A/E lesion formation) also initiates this phenotype and is consistent with a requirement for MyD88. Together our studies show that Ub⁺ aggregates form in vivo during enteric infection and reveal that this phenotype is dependent on both bacterial and host factors.
Fig. 6. NOX1 expression in the intestinal epithelia of mice infected with C. rodentium is induced in a MyD88-dependent manner and correlates with formation of Ub⁺ aggregates. a Representative immunofluorescence Mirax scanning micrographs of intestinal epithelial cells of C57BL/6 and MyD88⁻/⁻ mice infected with wild-type C. rodentium for 6 days. Tissue was co-stained for NOX1 (green) and a nuclear DAPI stain (blue). Note the increase in NOX1 in epithelial cells surrounding the infected intestinal lumen of the C57BL/6 mice compared to the MyD88-deficient mice. b Representative immunofluorescence micrographs of intestinal epithelial cells of C57BL/6 and MyD88⁻/⁻ mice infected with wild-type C. rodentium for 6 days. Tissue was triple stained for NOX1 (green), Ub⁺ aggregates (red) and a nuclear DAPI stain (blue). An increase in NOX1 signal is evident in the infected wild-type C57BL/6 mice. The increase in NOX1 signal correlates with the formation of Ub⁺ aggregates (arrows). NOX1 signal is instead diffuse in the MyD88-deficient mice, as is the absence of Ub⁺ aggregates. Unless otherwise indicated, scale bars = 10 μm.
The mechanism by which *C. rodentium* causes the formation of Ub² aggregates in epithelial cells is not clear. It is thought that the aggregates are formed by the congregation of defective ribosomal products, or DRiPs, that are ubiquitinated and shuttled into large cytosolic aggregates [39]. LPS treatment of dendritic cells has also been shown to induce a burst of protein translation, concomitant with Ub² aggregate formation [39]. A recent study by Netzer et al. [63] observed that cells exposed to virus, TLR ligands and oxidative stress displayed a 10-fold increase in translational infidelity. This finding suggests that stress can lead to changes in the amino acid code which may, in part, explain why DRiPs increase during times of cellular stress. We propose that the translational burst mediated...
by MyD88 signaling in response to intestinal bacterial infection may play a part in the occurrence of Ub\(^+\) aggregates in epithelial cells, due to an increased amount of polyubiquitinated proteins being sorted into these structures. MyD88-dependent induction of NOX1 (fig. 6) and subsequent ROS generation may also contribute to protein misfolding and Ub\(^+\) aggregate formation. Indeed, our results indicate that NOX1 expression is critical for Ub\(^+\) aggregate formation (fig. 7). The mechanism(s) by which NOX1-derived ROS results in Ub\(^+\) aggregate formation remains unknown.

Ub\(^+\) aggregates are thought to contribute to immune responses, possibly by playing a role in antigen processing and presentation where they act as cellular antigen storage depots, delaying degradation so that antigen presentation can be properly coordinated [33, 34, 62, 64]. However, it cannot be ruled out that Ub\(^+\) aggregate formation occurs as a consequence of other cellular immune responses such as a translational burst or ROS production. In either context, the fact that we can visualize these structures makes them a valuable tool to examine host-pathogen interactions at the level of fluorescence microscopy. Future studies in this area should focus on the mechanism of control in formation of Ub\(^+\) aggregates on a cellular level and the proteins targeted to these subcellular structures. Furthermore, it will be interesting to see if pathologically these structures are visible in other infection models. Our studies reinforce the idea that the formation of Ub\(^+\) aggregates is an important cellular response to infection, and worthy of future studies.

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