Cutting Edge: Microbial Products Elicit Formation of Dendritic Cell Aggresome-Like Induced Structures in Macrophages

Veronica Canadien, Tracy Tan, Rachelle Zilber, Jason Szeto, Andrew J. Perrin and John H. Brumell

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In response to a maturation stimulus, dendritic cells undergo the formation of ubiquitinated protein aggregates known as dendritic cell aggresome-like induced structures (DALIS). DALIS are thought to act as Ag storage structures, allowing for the prioritized degradation of proteins during infection. In this study, we demonstrate that murine macrophages can also form ubiquitinated protein aggregates that are indistinguishable from DALIS. These were formed in a dose- and time-dependent manner, and in response to a variety of microbial products. Surprisingly, the proteasome did not accumulate on these ubiquitinated protein structures, further underlining the difference between DALIS and aggresomes. Our studies suggest that DALIS formation is important for the function of Ag-presenting immune cells during infection.


**Materials and Methods**

**Cell culture**

For isolation of dendritic cells, whole bone marrow was isolated from the leg bones of NOR mice, and cells were grown with 10 ng/ml GM-CSF and 100 ng/ml IL-4 for 6 days. Dendritic cells were then purified by positive selection on CD11c beads (Miltenyi Biotec), as described by the manufacturer. Macrophages were isolated by growth of bone marrow cells with 10 ng/ml GM-CSF for 7 days. We are grateful to Dr. D. Spurrell and Dr. J. Danska (Hospital for Sick Children) for providing these cells for our studies. Rat embryonic hippocampal cell cultures were isolated from hippocampi of 18-day-old fetal rat brains. Dissection and removal of the hippocampus was described previously. (8) Hippocampal cells were plated and maintained in culture as described (9). We are grateful to C. Tsang and Dr. B. Trimble (Hospital for Sick Children) for providing these cells for our studies. RAW 264.7 and J774A.1 murine macrophages, and HeLa human epithelial cells were obtained from the American Type Culture Collection. All cells were cultured in DMEM plus 10% FBS without antibiotics and seeded in 24-well tissue culture plates on glass coverslips 16–24 h before use. At time of assay, growth medium was aspirated and replaced with fresh medium containing the appropriate agents.

**Cutting Edge: Microbial Products Elicit Formation of Dendritic Cell Aggresome-Like Induced Structures in Macrophages**

Veronica Canadien, * Tracy Tan, † Rachelle Zilber, † Jason Szeto, * Andrew J. Perrin, ‡ and John H. Brumell* ‡⁎

Dendritic cells are professional APCs, and play a critical role in both innate and adaptive immunity (1). In their immature state, dendritic cells migrate from the bone marrow to both lymphoid and nonlymphoid tissues. In response to microbial products or proinflammatory cytokines, dendritic cells undergo a maturation process that includes a variety of functional and phenotypic changes (1). As first demonstrated by Pierre and colleagues (2–4), this maturation process includes the formation of large polyubiquitinated protein aggregates, named dendritic cell aggresome-like induced structures (DALIS). Although these structures resemble aggresomes (5), they are transient in nature, do not localize to the microtubule organizing center, and are not caged with vimentin (2). DALIS are thought to contain misfolded ribosomal products (2), which can account for up to 30% of newly synthesized proteins (6). DALIS contain components of the ubiquitin system, suggesting that ubiquitination of misfolded proteins occurs in these structures. Evidence suggests that the ubiquitinated proteins contained in DALIS have a much longer half-life than those present in the cytosol (3). Thus, DALIS are thought to act as Ag storage centers during dendritic cell maturation, allowing for the prioritized degradation of proteins in response to infection (2, 3).

To date, DALIS formation has been observed only in dendritic cells. Because macrophages share a common hemopoietic precursor (7) and likewise regulate immune responses with the presentation of microbial Ags, we decided to examine ubiquitinated protein dynamics in this cell type in response to inflammatory stimuli. In this study, we show that macrophages can also induce DALIS formation in response to microbial products, suggesting that this phenotype is important for the function of Ag-presenting immune cells during infection.

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2 Address correspondence and reprint requests to Dr. John H. Brumell, IIIIR Program, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada, M5G 1X8. E-mail address: john.brumell@sickkids.ca

3 Abbreviations used in this paper: DALIS, dendritic cell aggresome-like induced structure; BHI, brain heart infusion.

4 The online version of this article contains supplemental material.
Bacterial strains, pathogen-associated molecular pattern agonists, and pharmacologic agents

Wild-type Salmonella typhimurium SL 1344 was used for these studies. For invasion by S. typhimurium, late-log bacterial cultures were used for infecting cells and prepared using a method optimized for bacterial invasion (10). Listeria monocytogenes strain LO28 was generously provided by Dr. P. Cossart (Pasteur Institute, Paris, France). S. typhimurium LPS and zymosan particles were from Sigma-Aldrich. LPS from Escherichia coli F470 was purified by hot water/phenol extraction and generously provided by Dr. C. Whitfield (Department of Microbiology, University of Guelph, Guelph, Ontario, Canada). Supernatant from L. monocytogenes was generated by overnight growth of L. monocytogenes strain LO28 in brain heart infusion (BHI) broth. Bacteria were removed by centrifugation, and the supernatant was filtered through a 0.2-μm filter. Latex beads were from Bangs Laboratories. Murine IFN-γ was purchased from R&D Systems.

A number of agents were tested for their ability to affect DALIS formation. For this, growth medium was replaced by fresh medium containing 100 ng/ml LPS, and one of the following drugs (all from Sigma-Aldrich) at the indicated concentration: cycloheximide (20 μg/ml), latrunculin B (10 μM), nocodazole (5 μM), and cytochalasin D (10 μM).

Immunofluorescence and confocal microscopy

Cells were fixed in 2.5% paraformaldehyde in PBS (pH 7.2) for 10 min at 37°C. Fixed cells were stained as previously described (10). FK2 mAbs and the affinity-purified rabbit polyclonal Abs to the 205 proteasome and Lmp7 were from Biomol. Rabbit polyclonal Abs to S. typhimurium (Salmonella O antigen) group B factors 1, 4, 5, and 12 were from DiCco Laboratories. mAbs to γ-tubulin were from Sigma-Aldrich. Samples were analyzed using a Zeiss Axiosvert microscope (×63 objective) and LSM 510 software. Confocal images were imported into Adobe Photoshop, converted to CMYK format, and assembled in Adobe Illustrator for labeling.

Enumeration of DALIS

A Leica DMIRE2 fluorescence microscope was used to visually quantify DALIS. For this, random fields of cells stained for ubiquitinated proteins with the FK2 mAb were selected. DALIS >0.5 μm in diameter were counted in each cell. For each treatment, at least 100 cells were counted. The average ± SD for at least three experiments is presented.

Live cell imaging

RAW 264.7 cells were transfected with GFP-ubiquitin (generously provided by Drs. S. Qian and J. Yewdell, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD; Ref. 11) and then treated with 1 μg/ml S. typhimurium LPS for 8 h. Cells were then mounted onto an inverted Leica DMIRE2 spinning disk confocal microscope with a heated stage and an Orca AG CCD camera. Approximately 27 confocal images were acquired at different focal planes (0.38-μm optical slices) through each cell at a given time point. Images were acquired every 90 s through the duration of each experiment. All images were obtained with cells kept at 37°C in RPMI 1640 medium with 1-glutamine buffered to pH 7.2 with HEPES.

Results and Discussion

To visualize ubiquitinated proteins, we immunostained cells with the FK2 mAb to mono- and polyubiquitinated proteins (12). In all untreated cell types, ubiquitinated proteins were diffusely localized in the cytosol and nucleus (Fig. 1). In response to LPS, DALIS were readily seen in murine bone marrow-derived dendritic cells, as previously described (2–4). Large aggregates of ubiquitinated proteins that resembled DALIS were also observed in bone marrow-derived macrophages and two macrophage cell lines, but not in rat brain neurons (Fig. 1). HeLa epithelial cells, or other LPS-nonresponsive cell types (data not shown).

Because the ubiquitinated protein aggregates in macrophages resembled DALIS, we characterized the former structures in RAW 264.7 cells, a murine macrophage cell line. The aggregates were formed in a dose-dependent manner in response to LPS from both S. typhimurium (Fig. 2A) and E. coli (B). These structures were dynamic, being most prevalent 8–10 h after LPS treatment and declining thereafter (Fig. 2C). The aggregates did not colocalize with γ-tubulin (Fig. 2D), which is a typical feature of ubiquitinated proteins present in aggresomes (5). In further contrast to aggresomes, the aggregates were not sensitive to nocodazole, which disrupts the microtubule cytoskeleton (Fig. 2E). Surprisingly, disruption of the actin cytoskeleton with cytochalasin D enhanced formation of the ubiquitinated protein aggregates (Fig. 2F). Similar results were seen with latrunculin B (data not shown). Treatment of cells with cycloheximide blocked appearance of the aggregates, indicating that protein synthesis is required for their formation (Fig. 2G). Interestingly, addition of cycloheximide to cells 8 h after LPS treatment led to the hollowing of DALIS structures after a 1- to 2-h period (data not shown). This suggests that protein turnover occurs in the center of these structures, and that newly synthesized proteins are targeted to this compartment. This observation is consistent with the observations of Pietre and colleagues (3), who demonstrated that newly synthesized proteins labeled with puromycin are delivered to the central region of DALIS. Finally, we visualized the ubiquitin-activating enzyme E1 on ubiquitinated protein aggregates in RAW 264.7 cells, suggesting that these structures were the site of protein ubiquitination (data not shown). Together, these data suggest that...
ubiquitinated protein aggregation can occur in macrophages by a mechanism indistinguishable from DALIS formation previously characterized in dendritic cells by Pierre and colleagues (2, 3). For simplicity, we will refer to the ubiquitinated protein aggregates formed in macrophages in response to microbial products as DALIS.

We examined DALIS formation in live cells by their expression of a GFP fusion to ubiquitin (11). RAW 264.7 cells were transiently transfected with GFP-ubiquitin and then treated for 8 h with LPS, followed by microscopic analysis. Using spinning disk confocal microscopy to acquire three-dimensional images at each time point (every 90 s), we observed rapid movement and fusion of DALIS within cells (supplemental video 1), consistent with the observations of Pierre and colleagues (3). Nocodazole treatment blocked movement of DALIS in cells (supplemental video 2), despite the fact that it does not block their formation (Fig. 2E). These findings demonstrate that DALIS are dynamic structures, capable of undergoing microtubule-based movement in APCs.

DALIS formation in macrophages was induced by exposure to a wide variety of microorganisms, including Gram-negative *S. typhimurium* and Gram-positive *L. monocytogenes* (data not shown). Exposure to intact bacteria was not necessary, because growth medium from bacterial cultures was sufficient to induce the DALIS phenotype (Fig. 3A). Bacterial growth medium (BHI) alone did not induce DALIS (Fig. 3B). We also observed DALIS formation in response to yeast zymosan (Fig. 3C). The DALIS structures did not appear to be preferentially localized near phagosomes containing these particles. Interestingly, DALIS were not observed in cells exposed to latex beads, commonly used as a phagocytic stimulus (Fig. 3D). We also observed little, if any, DALIS formation in response to the cytokine IFN-γ, a potent activator of macrophages (Fig. 3E).
These findings suggest that DALIS formation occurs specifically in response to microbial products and pathogen-associated molecular patterns.

The proteasome plays a central role in the degradation of misfolded proteins in eukaryotic cells and generates peptides for presentation on MHC class I (6). Recent studies suggest that, in response to infection, the proteasome can undergo subcellular relocalization to allow for the degradation of foreign proteins. For example, the proteasome has been localized to nascent aggresomes in macrophages, and is thought to degrade proteins present in the cytosol of macrophages and not residing within their characteristic Salmonella-containing vacuole (16). Recruitment of the proteasome to bacteria in the cytosol was visualized using polyclonal Abs to the 20S proteasome, and is depicted in Fig. 4A. By contrast, the proteasome did not accumulate on DALIS at any time point that we examined (Fig. 4B). Similarly, the inducible proteasome subunit Lmp7 did not accumulate at DALIS after IFN-γ/LPS treatment (Fig. 4C). These findings further highlight the difference between DALIS and aggresomes, because the latter structures contribute to cellular toxicity, in part, through their accumulation and inhibition of proteasomes (17). Furthermore, this supports the notion that ubiquitinated proteins present in DALIS are protected from degradation by the proteasome (3).

Our studies reveal several important findings. First, DALIS formation can occur in macrophages and is not unique to dendritic cells, as was previously thought. This is significant, because macrophages are the host cell niche exploited by a number of intracellular bacterial pathogens. We have also demonstrated that the proteasome is not recruited to DALIS, despite the fact that the proteasome is normally recruited to large structures containing ubiquitinated proteins such as aggresomes (17) and cytosolic bacteria (16). This is consistent with the findings of Pierre and colleagues (3), who used biochemical and microscopic approaches to demonstrate that the ubiquitinated proteins present in DALIS have a much longer half-life than those present in the cytosol. Together, these findings support the hypothesis that DALIS can serve as Ag storage structures (2). The storage of misfolded “self” proteins during infection may allow for efficient presentation of peptides from “foreign” microbial proteins. Indeed, relocalization of the proteasome to phagosomes (13) and bacteria in the cytosol (16) may promote the generation and presentation of foreign peptides. Finally, we have demonstrated that DALIS are mobile structures capable of undergoing fusion. Microtubule-dependent movement (but not formation) of DALIS implies that the storage of misfolded proteins is spatially regulated within the cell.

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### Disclosures

The authors have no financial conflict of interest.

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