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Research Paper

Induction of Live Cell Phagocytosis by a Specific Combination of Inflammatory Stimuli

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ABSTRACT

Conditions of severe hyper-inflammation can lead to uncontrolled activation of macrophages, and the ensuing phagocytosis of live cells. However, relationships between inflammatory stimuli and uncontrolled phagocytosis of live cells by macrophages are poorly understood. To identify mediators of this process, we established phagocytosis assays of live cells by stimulating macrophages with CpG DNA, interferon-γ, and anti-interleukin-10 receptor antibody. In this model, various cell surface receptors were upregulated on macrophages, and phagocytosis of live cells was induced in a Rac1-dependent manner. Subsequent inhibition of the ICAM-1, VCAM-1, and both of these receptors abolished *in vitro* and *in vivo* phagocytosis of live T cells, myeloid cells, and B cells, respectively. Specifically, the reduction in lymphocyte numbers due to *in vivo* activation of macrophages was ameliorated in *Icam-1*-deficient mice. In addition, overexpression of ICAM-1 or VCAM-1 in non-phagocytic NIH3T3 cells led to active phagocytosis of live cells. These data indicate molecular mechanisms underlying live cell phagocytosis induced by hyper-inflammation, and this experimental model will be useful to clarify the pathophysiological mechanisms of hemophagocytosis and to indicate therapeutic targets.

1. Introduction

In our body, billions of cells perform apoptotic cell death every day and are removed by phagocytes such as macrophages. This process is known as efferocytosis and is dependent on recognition and selective phagocytosis of dead cells by phagocytes (Nagata et al., 2010). Among plasma membrane molecules, the phospholipid phosphatidylserine is important for phagocyte recognition of cells to engulf (Segawa and Nagata, 2015). Hence, in live cells, phosphatidylserine is exclusively maintained in the inner leaflet of the lipid bilayer through the actions of phospholipid flippases (Segawa et al., 2014). During apoptosis, phospholipid flippases are inactivated and phospholipid scramblases are activated, leading to exposure of phosphatidylserine to the outer leaflet (Suzuki et al., 2013). Subsequently, phagocytic recognition and signaling is mediated by phosphatidylserine-binding proteins such as MFG-E8, Tim4, BAI1, and Gas6 (Nagata et al., 2010). In addition, cell surface expression of CD31 or CD47 on live cells is protective against phagocytosis, and these molecules are downregulated during apoptosis (Brown et al., 2002; Gardai et al., 2005). Together these mechanisms prevent phagocytosis of live cells and ensure phagocytosis of apoptotic cells.

In contrast, phagocytosis of live cells can be induced by uncontrolled activation of macrophages by strong immunologic conditions such as systemic infection, autoimmunity, and malignancy. Specifically, phagocytosis of live blood cells and their precursors by macrophages is known as hemophagocytosis. It is often observed in hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS), which are characterized by overwhelming immune activation and excessive production of inflammatory cytokines (Janka, 2012; Grom et al., 2016). Several studies have defined hemophagocytosis as a type of efferocytosis. For example, CD47 expression was reportedly downregulated in live hematopoietic stem cells from HLH patients (Kuriyama et al., 2012). Moreover, numbers of apoptotic erythrocytes were increased in a mouse model of HLH (Ohyagi et al., 2013). However, because abnormal activation of macrophages by inflammatory cytokines such as interferon-γ (IFN-γ) is the primary cause of hemophagocytosis (Zoller et al., 2011), these observations offer little to the understanding of pathogenic mechanisms. In particular, it remains unclear how inflammatory cytokines induce macrophages to engulf live cells.

We therefore considered that establishment of an *in vitro* cell culture model of live cell phagocytosis will be useful not only for identifying phagocytic receptors of live cells but also for clarifying the pathogenesis of hemophagocytosis to indicate therapeutic targets. However, specific *in vitro* stimuli inducing phagocytosis of live cells by macrophages remain elusive. Recently, repeated injections of Toll-like receptor (TLR)

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9 ligand, CpG DNA into mice have been reported to induce MAS-like diseases and hemophagocytosis (Behrens et al., 2011). Accordingly, we tried to induce phagocytosis of live cells in cultured macrophages using CpG DNA treatments.

2. Materials and Methods

2.1. Mice, Cells, and Reagents

C57BL/6 mice were purchased from SLC, Japan and C57BL/6-Tg (CAG-EGFP) mice (Okabe et al., 1997) were a kind gift from M. Okabe. *Icam*-1 KO mice were generated by pronuclear injection of CRISPR/Cas9 pX330 vectors targeting mouse *Icam-1* exon-4 (CGCTGCGTTTTGGAGCTAGCGG) and exon-6 (TCCTAAGATGACCTGCAGACGG) to introduce frameshift mutations (PAM sequences are underlined). All mice were housed in a pathogen-free facility and all animal experiments were performed according to protocols that were approved by the Animal Research Committee of Kanazawa University, Japan. Bone marrow-derived macrophages (BMDMs) were generated by culturing bone marrow cells from femurs and tibias of mice for 4–6 days in high glucose DMEM (Nacalai, Japan) supplemented with 10% FBS (Biowest), 1% penicillin/streptomycin, and 10 units/ml of macrophage colony-stimulating factor (M-CSF), which was prepared using conditioned medium from human M-CSF overexpressing mouse L929 cells (Takeshita et al., 2000). Primary cultures of thioglycollate-elicited peritoneal macrophages (pMACs) and bone marrow-derived dendritic cells (BMDCs) were prepared as described previously (Hanayama et al., 2002; Miyasaka et al., 2004). All cell lines were obtained from RIKEN Bio Resource Center (Japan) and tested for mycoplasma contamination. Cells were treated either with recombinant IFN-γ, the Rac1 inhibitor NSC23766 (Wako, Japan), cycloheximide (Nacalai, Japan), CpG ODN-1826 (5′- TCCATG ACGTTCCTGACGTT-3′, Hokkaido System Science, Japan), or BAPTA-AM (Dojindo, Japan). Monoclonal antibodies against mouse B220 (RA3-6B2, RRID:AB_312996), CD3 (17A2, RRID:AB_312661), CD4 (GK1.5, RRID:AB_312696), CD8a (53-6.7, RRID:AB_312751), CD11b (M1/70, RRID:AB_312794), CD68 (FA11, RRID:AB_10575475), Gr-1 (RB6-8C5, RRID:AB_313368), ICAM-1 (YN1/1.7.4, RRID:AB_313700), IL-10R (1B1.3a, RRID:AB_313521), Integrin α_V (RMV-7, RRID:AB_2265155), Integrin β_3 (2C9.G2, RRID:AB_313086), LAMP-1 (1D4B, RRID:AB_572003), LFA-1α (M17/4, RRID:AB_10694867), PECAM-1 (MEC13.3, RRID:AB_312918), VCAM-1 (429, RRID:AB_313209), VLA-4α (9C10, RRID:AB_2129608), and VLA-5α (5H10-27, RRID:AB_313065), and rat IgG1κ (RTK2071, RRID:AB_326519), IgG2a κ (RTK2758), IgG2b κ (RTK4530, RRID:AB_2086803), and Armenian hamster IgG (HTK888) isotype control antibodies, and mouse IL-10 ELISA Kit were purchased from BioLegend. The D89E mutant of mouse MFG-E8 was prepared as described previously (Hanayama et al., 2002).

2.2. Plasmids and Transfection

DNA fragments for full length coding sequences of mouse *Icam-1* and *Vcam-1* were prepared using RT-PCR after RNA extraction from CpG, IFN-γ, and αIL-10 cotreated BMDMs using the following primers: ICAM-1-Fw, 5′-CCCGGATCCCTACCATGGCTTCAACCCGT-3′ and ICAM-1-Rv, 5′-AAAGCGGCCGCTCAGGGAGGTGGGGC-3′ (*Bam*HI and *Not*I sites are underlined), and VCAM-1-Fw, 5′-ATTAATTAAGCCACCATGCCTGTGAAGAGGTC-3′ and VCAM-1-Rv, 5′-AAAGCGGCCGCCTACACTTTGGATTTCTGTGC -3′ (*Pac*I and *Not*I sites are underlined). PCR fragments were digested using *Bam*HI/*Pac*I and *Not*I restriction endonucleases, and were inserted into pMXs retrovirus vectors (Kitamura et al., 2003). The pMXs-Rac1 construct was a gift from S. Nagata. Retroviral plasmid lipofection into PLAT-E packaging cells (Morita et al., 2000) was performed using FuGENE6 (Promega), and 48 h later, NIH3T3 cells or BMDMs were infected with culture supernatants in the presence of 10 μg/ml polybrene to establish stable transformants. NIH3T3 transformants expressing integrin $\alpha_V\beta_3$ were generated as described previously (Hanayama et al., 2002).

2.3. In Vitro Phagocytosis Assays

Phagocytes (1×10^5 cells) were cultured on 24-well plates (Corning) for flow cytometric analyses or on NUNC Lab-Tek II 8-well chamber glass slides (Thermofisher) for microscope analyses, and were then activated using various combinations of IFN-γ (100 U/ml), CpG ODN-1826 (0.5 μg/ml), and/or αIL-10R (1.25 μg/ml) in the absence or presence of cycloheximide (1 μg/ml) or the Rac1 inhibitor NSC23766 at 50, 100, or 200 μM for 20 h. Prey cells such as thymocytes, splenocytes, and myeloid cells were freshly prepared from 4 to 6 week old C57BL/6 mice. Myeloid cells were prepared from bone marrow by depleting T and B cells using a FACSAria cell sorter (BD Biosciences) with αCD3 and αB220 antibodies. Apoptosis was induced in thymocytes using 10 μM dexamethasone treatments for 4 h, and in myeloid cells by UV irradiation at 200 J/cm^2 and incubation for 2 h. For flow cytometric analyses, prey cells were washed twice with PBS and were incubated for 30 min with 1 μM CellTracker green dye (CMFDA) (Thermofisher). Reactions were stopped by adding 1 ml of FBS and the cells were then washed twice with DMEM containing 10% FBS. The labeled cells (1×10^6 cells) were added to BMDMs that had been pre-treated with or without 4 μg/ml antibodies, 10 or 20 μM BAPTA-AM, or 7 μg/ml MFG-E8 D89E for 30 min. Phagocytosis proceeded for 2.5 h at 37 °C and prey cells that remained free were removed by washing twice with PBS. Phagocytes were collected from the plates by trypsinization, and were washed and suspended in PBS containing 2% FBS and 0.02% NaN3. Percentage phagocytosis was determined in triplicates by quantifying the percentage of CMFDA-positive phagocytes using FACSVerse (BD Biosciences). For microscope analyses, prey cells (1×10^6 cells) were added to GFP-labeled phagocytes on glass slides that had been pre-treated with or without antibodies at 4 (for BMDMs) or 10 μg/ml (for NIH3T3 cells, pMACs, or BMDCs) for 30 min. In some experiments, prey cells were pre-labeled with 0.1 μg/ml pHrodo Red (Thermofisher). After co-culturing for 2.5 h, cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA)/PBS. When co-cultured with splenocytes, the phagocytes were permeabilized with ice-cold acetone and were stained with APC αB220, αCD4, or αCD8a antibodies in PBS containing 1% BSA to identify cell types of the engulfed cells. The cells were then mounted with ProLong Gold Antifade (Thermofisher) containing 1% DAPI (Dojindo, Japan), and were observed using an FV10i confocal microscope (Olympus, Japan). Engulfed cells can be detected as dark spots in the cytoplasm and total numbers of engulfed cells of 200 phagocytes (50 cells/field × 4 fields) were counted blindly, and phagocytosis indexes were determined as average numbers of engulfed cells per 100 phagocytes.

2.4. Detection of Live and Apoptotic Cells

TUNEL, JC-1, or Annexin V staining was performed using ApopTag Fluorescein Direct *In Situ* Apoptosis Detection Kits (Merck Millipore), MitoPT JC-1 Assay Kit (ImmunoChemistry Technologies), or FITC-Annexin V (BioLegend), respectively. For electron microscope analyses, BMDMs were cultured on Cell Desk (Sumitomo Bakelite, Japan) polystyrene cover slips. After phagocytosis, cells were fixed using 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium-phosphate buffer (pH 7.4) and were washed three times for 5 min in the same buffer. Cells were then post-fixed for 1 h in 0.1 M sodium-phosphate buffer (pH 7.4) containing 1% osmium tetroxide and 1% potassium ferrocyanide, and were then dehydrated in a graded series of ethanol and embedded in Epon812 (TAAB, UK). Subsequently, 80 nm ultra-thin sections were stained with a saturated solution of uranyl acetate and lead citrate. Electron micrographs were obtained using a JEM-1011 transmission electron microscope (JEOL, Japan).

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