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Protein S and Gas6 induce efferocytosis of HIV-1-infected cells

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ABSTRACT

Efferocytosis, the phagocytic clearance of apoptotic cells, can provide host protection against certain types of viruses by mediating phagocytic clearance of infected cells undergoing apoptosis. It is known that HIV-1 induces apoptosis and HIV-1-infected cells are efferocytosed by macrophages, although its molecular mechanisms are unknown. To elucidate the roles that efferocytosis of HIV-1-infected cells play in clearance of infected cells, we sought to identify molecules that mediate these processes. We found that protein S, present in human serum, and its homologue, Gas6, can mediate phagocytosis of HIV-1-infected cells by bridging receptor tyrosine kinase Mer, expressed on macrophages, to phosphatidylserine exposed on infected cells. Efferocytosis of live infected cells was less efficient than dead infected cells; however, a significant fraction of live infected cells were phagocytosed over 12 h. Our results suggest that efferocytosis not only removes dead cells, but may also contribute to macrophage removal of live virus producing cells.

1. Introduction

Phagocytosis of dying or dead cells by phagocytes such as macrophages is known as efferocytosis (Birge et al., 2016; Zent and Elliott, 2017). Removal of dead or dying cells by efferocytosis is critical for maintaining homeostasis and preventing inflammation and autoimmune reactions (Elliott and Ravichandran, 2016). Because infection by certain viruses induces massive cell death, efferocytosis plays an important role in maintaining host homeostasis during viral infection (Jorgensen et al., 2017; Nainu et al., 2017). Viral infection induces changes of cell surface molecules related to cell death, and macrophages can recognize these changes and engulf the affected cells. It has been reported that macrophages perform efferocytosis of cells infected with the influenza (Fujimoto et al., 2000; Hashimoto et al., 2007; Shiratsuchi et al., 2000; Watanabe et al., 2005, 2002, 2004; Mukherjee et al., 2017), papilloma (Hermetet et al., 2016), and Drosophila C viruses (Nainu et al., 2015; Nonaka et al., 2017), as well as Lymphocytic choriomeningitis virus (LCMV) (Alatery et al., 2010), and Human Immunodeficiency Virus Type 1 (HIV-1) (Akbar et al., 1994).

Efferocytosis can also play a role in protecting the host from pathogens (Martin et al., 2014; Nainu et al., 2015; Tufail et al., 2017). When cells are infected with certain types of viruses, the cells undergo apoptosis to prevent pathogens from exploiting the host cell machinery. Efferocytosis then mediates phagocytic clearance of the infected cells and prevents further production of viruses.

The roles of efferocytosis in protection against pathogens have been well-studied for influenza virus infection (Fujimoto et al., 2000; Hashimoto et al., 2007; Shiratsuchi et al., 2000; Watanabe et al., 2005, 2002, 2004). When cells are infected with influenza virus, they start exposing phosphatidylserine (PtdSer), which is mainly present in the inner layers of the cell membrane but becomes exposed when cells undergo apoptosis. Macrophages and neutrophils efferocytose infected cells by recognizing exposed PtdSer. By clearing the infected cells, efferocytosis inhibits virus spread.

HIV-1 infection is known to induce exposure of PtdSer by inducing cell death (apoptosis or pyroptosis) (Terai et al., 1991; Doitsh et al., 2014). Analysis of tissue sections from HIV-1-infected patients has shown that macrophages engulf HIV-1-infected T-cells by efferocytosis (Akbar et al., 1994). *In vitro* studies showed that macrophages can recognize and engulf HIV-1-infected cells by a mechanism that is independent of viral envelope proteins and antiviral antibodies (Baxter et al., 2014). Although the molecular mechanisms by which

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macrophages selectively capture and engulf apoptotic HIV-1-infected cells are not known, it is likely that recognition of PtdSer plays a role.

Phagocytes recognize PtdSer on dead cells by various molecular mechanisms that can be largely categorized as mediated by soluble molecules that bridge dead cells and phagocytes, including protein S, Gas6, and MFG-E8 (Hafizi and Dahlback, 2006; Hanayama et al., 2002), or mediated by the receptors that directly bind PtdSer, including TIM-1, -3, and -4, CD300a, BAI-1, RAGE, and Stabilin 1 and 2 (DeKruyff et al., 2010; Friggeri et al., 2011; He et al., 2011; Kobayashi et al., 2007; Miyanishi et al., 2007; Nakahashi-Oda et al., 2012; Park et al., 2007, 2009; Simhadri et al., 2012). In this study, we found that protein S/Gas6 can mediate phagocytosis of HIV-1-infected cells by bridging PtdSer exposed on the infected cells to one type of receptor tyrosine kinase, Mer, which is expressed on macrophages. We investigated whether this efferocytosis mechanism can inhibit virus production by engulfment of infected cells producing virus.

2. Results

2.1. HIV-1 infection induces PtdSer exposure

Because HIV-1 infection is known to induce exposure of PtdSer on infected cells, we hypothesized that macrophages capture infected cells by recognizing exposed PtdSer, similar to how they recognize influenza virus-infected cells (Fujimoto et al., 2000; Hashimoto et al., 2007; Shiratsuchi et al., 2000; Watanabe et al., 2005; Watanabe et al., 2002; Watanabe et al., 2004).

We first investigated the time-course of Gag (HIV-1 p24) expression, Env expression, PtdSer exposure, cell death, and virus production to determine whether exposed PtdSer can be a marker for phagocytes to recognize HIV-1-infected cells (Fig. 1A). For target cells, we used MT4CCR5, a CD4+ T-cell line ectopically expressing CCR5. Since nearly 100% of MT4CCR5 cells become infected within two days post-infection (Fig. 1A), this cell line provides an ideal model for experiments to investigate the molecular mechanisms of efferocytosis of HIV-1-infected cells.

MT4CCR5 cells were infected with X4-tropic strain of HIV-1 (HIV-1_{NL4-3}). Cells infected with virus expressed Gag and Env at low levels one day post-infection and then at drastically increased levels two days post-infection (Fig. 1A). PtdSer exposure, which was analyzed by Annexin V (ANX V) staining, started two days post-infection and increased until four days post-infection. The cells infected with heat-inactivated virus do not expose PtdSer (Fig. S1), indicating that exposed PtdSer can be a marker for macrophages to recognize HIV-1-infected cells. Cell death also started at two days post-infection (~20%) and drastically increased at 3 days post-infection (~65%). When cell death and Env expression of infected cells were analyzed together (Fig. 1B and Fig. S1), Env expression was lower in dead cells than live cells (Fig. 1B). When cell death and PtdSer exposure were analyzed together, PtdSer exposure was higher on dead cells than live cells (Fig. 1B).

Progeny virus production started between one and two days post-infection, peaked between two and three days post-infection, and drastically decreased between three and four days post-infection (Fig. 1C). Time course analyses of cell death and virus production suggested that high viability is required for efficient production of infectious virus.

2.2. Expression of PtdSer receptors on macrophages

We next investigated whether macrophages express PtdSer receptors. We obtained macrophages by differentiating peripheral blood monocytes. To avoid the effects of protein S present in serum, we depleted bovine protein S from fetal calf serum used in this study by barium chloride precipitation (Bhattacharyya et al., 2013).

We recently investigated the PtdSer recognition mechanisms that can efficiently recognize exposed PtdSer (Morizono and Chen, 2014; Morizono et al., 2011). Our results showed that protein S/Gas6, which bridges PtdSer to TAM receptors (TYRO 3, Axl, and Mer) on phagocytes, and TIM-1 and -4, which bind PtdSer directly, bind PtdSer most efficiently. To investigate whether any of these molecular mechanisms can mediate phagocytosis of HIV-1-infected cells by macrophages, we first analyzed expression of these receptors on macrophages and found only Mer was expressed on macrophages (Fig. 1D). We also analyzed expression of those five receptors on macrophages differentiated under various culture conditions, including use of granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), or no cytokines, or using medium supplemented with normal human or fetal bovine serum or serum-free medium, or in the presence and absence of Dexamethasone. Of the five receptors, only Mer was expressed on macrophages under any conditions tested (data not shown).

2.3. Human serum induces phagocytosis of infected MT4CCR5 cells

Because Mer can mediate efferocytosis through an interaction with protein S in serum, we investigated whether human serum can induce phagocytosis of HIV-1-infected cells. We used a pH-sensitive fluorescent dye, pHrodo Red, to analyze phagocytosis of cells, as previously described (Aziz et al., 2013). We labeled MT4CCR5 cells or the cells infected with HIV-1_{NL4-3} (MT4CCR5/NL4-3) with pHrodo Red so that they emit fluorescent signals upon exposure to the low-pH environment in the phagosomes of phagocytes. We used MT4CCR5/NL4-3 cells at three days post-infection to investigate phagocytic mechanisms. We co-cultured macrophages with pHrodo Red-labeled MT4CCR5 or MT4CCR5/NL4-3 cells in the absence or presence of human serum, using macrophages obtained from four donors. The gating strategy to quantitate phagocytosis by flow cytometry analysis is shown in Fig. 2A. In the absence of serum, percentages of pHrodo Red signal-positive macrophages co-cultured with MT4CCR5/NL4-3 were higher than those co-cultured with MT4CCR5 (Fig. 2B), indicating that macrophages can preferentially engulf HIV-1-infected cells independently of serum, albeit at low efficiency. Human serum enhanced phagocytosis of MT4CCR5/NL4-3 more efficiently than for MT4CCR5 cells (Fig. 2B), demonstrating that human serum preferentially induces phagocytosis of HIV-1-infected cells by macrophages. We confirmed that four lots of human AB serum could induce phagocytosis of MT4CCR5/NL4-3 cells by macrophages (data not shown). After confirmation, we used the same lot throughout this study.

We next confirmed the flow cytometric analysis of phagocytosis, using quantitative analysis of microscopic images by imaging flow cytometry. To analyze binding of macrophages to prey cells, we tracked MT4CCR5 and MT4CCR5/NL4-3 cells by labeling them with CellTrace Violet fluorescent dye, co-cultured the cells with macrophages in the presence or absence of human serum, then stained cells with APC-conjugated antibody against CD14 to identify macrophages. In the absence of serum, 1% and 3.6% of macrophages bound MT4CCR5 and MT4CCR5/NL4-3 cells, respectively (Fig. 2C), demonstrating that macrophages can recognize HIV-1-infected cells independently of serum, which is consistent with the results using pHrod Red labeling and flow cytometry. Human serum enhanced binding of macrophages to MT4CCR5/NL4-3 (from 3.6% to 40.7%), but only minimally to MT4CCR5 (from 1% to 1.1%) (Fig. 2C). To distinguish macrophage internalization of prey cells from macrophage:prey cell binding, we employed imaging flow cytometry analysis software, IDEAS 6.2, which can quantitate internalization of violet signals into macrophage APC signals as internalization scores. Events with internalization scores of less than 0 indicate that MT4CCR5/NL4-3 cells (violet color) bound the surfaces of macrophages (red color) (Fig. 2C), and those with internalization scores greater than 0 indicate internalization of the MT4CCR5/NL4-3 signal into macrophages (Fig. 2C). We used an internalization score of 1.5 as a threshold to stringently identify engulfment of cells, and calculated percentages of macrophages that had

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