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Immature human DCs efficiently translocate endocytosed antigens into the cytosol for proteasomal processing

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ABSTRACT

Cross-presentation of endocytosed antigen is essential for induction of CD8 effector T cell responses and a hallmark of dendritic cells (DCs). The mode of antigen processing in this context is controversial and some models imply translocation of the antigen from the endosomes into the cytosol. To test this hypothesis we made use of the pro-apoptotic properties of cytochrome c when in the cytosol, and confirmed that it indeed triggered apoptosis of human immature DCs but only at high concentrations. Proteasome inhibitors reduced the required concentration of cytochrome c thousand-fold, indicating that protein translocated into the cytosol is rapidly degraded by proteasomes. Mature DCs were also susceptible to cytochrome c-triggered apoptosis at high concentrations but proteasome inhibitors did not increase their sensitivity. Other cross-presenting cells such as B cells and monocytes were not sensitive to cytochrome c at all, indicating that they do not shuttle internalized antigen into the cytosol. Thus, processing of internalized antigens seems to follow different pathways depending on cell type and, in case of DCs, maturation state. Immature DCs appear to have a unique capacity to shuttle external antigen into the cytosol for proteasomal processing, which could explain their efficiency in antigen cross-presentation.

1. Introduction

Presentation of exogenous antigens on MHC class I molecules to CD8+ T cells, termed cross-presentation, is key to the induction of cytolytic T lymphocytes to eliminate tumor and infected cells (Berard et al., 2000; Joffre et al., 2012). Cross-presentation is a capacity of 'professional' antigen presenting cells (APCs), *i.e.* B lymphocytes, monocytes/macrophages and dendritic cells (DCs) (Jung et al., 2002). For that, the APCs must first take up antigens, process them and load the resulting peptides onto MHC class I molecules to be presented at the surface for recognition by CD8+ T cells. The cellular mechanisms involved in this process are controversial. The superior ability of DCs for cross-presentation is thought to be in part due to a unique intracellular pathway for translocation of Ag from endosomes into the cytosol for access to the conventional MHC class I processing pathway (Rodriguez et al., 1999).

Cytochrome c (cyt c) is a 13-kDa mitochondrial protein and component of the respiratory chain (Dennerlein and Rehling, 2015). Cyt c may induce apoptosis when released into the cytosol, which is the so-called mitochondrial intrinsic pathway of apoptosis (Green and Kroemer, 2004; Schafer and Kornbluth, 2006). In the cytosol it binds to the apoptotic protease-activating factor 1 (Apaf-1) to form a procaspase-9-activating protein complex called apoptosome (Schafer and Kornbluth, 2006) resulting in cleavage and activation of the effector caspases 3 and 7 that then degrade a range of intracellular proteins leading to cell death. Cells from mice deficient of Apaf-1 are not susceptible to cyt c-induced apoptosis, indicating Apaf-1 as the major mediator of cyt c-induced apoptosis (Hao et al., 2005).

Lin et al. (Lin et al., 2008) reasoned that, if cross-presenting DCs translocate internalized molecules into the cytosol, exogenous cyt c should induce apoptosis selectively in Apaf-1 positive cross-presenting DCs. Their findings showed that cyt c at high concentrations is toxic to the cross-presenting CD8α+ but not to CD8α- DCs *in vitro* and *in vivo* suggesting that only in CD8α+ cyt c was translocated into the cytosol to trigger apoptosis. Since Lin's work, other groups have used the cyt c model to study cross-presentation by DCs (Farrand et al., 2009; Cockburn et al., 2011; Imai et al., 2011; Segura et al., 2013). However, very high concentrations of cyt c above 5 mg/mL are required (Lin

Abbreviations: Ag, antigen; Apaf-1, apoptotic protease-activating factor 1; APC, antigen presenting cell; CD, cluster of differentiation; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; HLA, human leukocyte antigen; iDC, immature dendritic cell; IL, interleukin; LPS, lipopolysaccharide; mDC, mature dendritic cell; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; RPMI, Roswell park memorial institute medium; XCR1+, x chemokine receptor 1

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et al., 2008; Farrand et al., 2009; Cockburn et al., 2011; Imai et al., 2011; Segura et al., 2013). Here we present data that explain why only high concentrations of soluble cyt c trigger apoptosis in human DCs.

2. Material and methods

2.1. DC generation and treatment

Human DCs were generated from plastic-adherent peripheral blood mononuclear cells (PBMCs) (Baleeiro et al., 2015). Briefly, PBMCs from healthy donors were isolated from buffy coats by Ficoll gradient centrifugation and plated in 75 cm^2 flasks for monocytes adherence. After 1–2 h, non-adherent cells were removed and the adherent cells cultured in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). GM-CSF (50 ng/mL; Genzyme, Cambridge, MA, USA) and IL-4 (50 ng/mL; PromoCell GmbH, Germany) were added on days 0 and 4. The cultures were maintained at 37 °C in a humidified atmosphere with 8% CO_2. For generation of mature DCs, 1 μg/mL LPS (Sigma-Aldrich, Germany) was added to immature DCs on day 5 and the culture continued. On day 5 (iDCs) or 7 (mDCs), horse cyt c (Sigma-Aldrich; St. Louis, MO, USA) was added at the indicated concentrations and the culture continued for 24 h. In some experiments, cells were incubated with lactacystin (Sigma-Aldrich, Steinheim, Germany) at 10 μg/mL, leupeptin and pepstatin A (all Sigma-Aldrich, Steinheim, Germany) at 20 μM and/or the caspase 3 inhibitor Z-DEVD-FMK (R & D Systems, Minneapolis, MN, USA) at 5 μM for 30 min prior to the addition of cyt c. The cells were then harvested and their viability analyzed by flow cytometry. To establish the maturation state of the DCs, the cells were analyzed for HLA-ABC, HLA-DR, CD80, CD83 and CD86 (BD Bioscience, Chicago, IL, USA). All flow cytometry was done with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were processed with the CellQuest (Becton Dickinson, San Jose, CA, USA) and WinMDi 2.9 (Purdue University, USA) softwares.

2.2. PBMCs culture and treatment

PBMCs from healthy donors were cultured for 7 days in RPMI-1640 culture medium (Invitrogen, Carlsbad, CA, USA) with 1% converted human serum (Pan Biotech, Germany). This protocol has been shown to induce differentiation of monocytes into macrophages (Eligini et al., 2013). On day 7, horse cyt c (Sigma-Aldrich; St. Louis, MO, USA) was added at the indicated concentrations, in the presence or absence of lactacystin (Sigma-Aldrich, Steinheim, Germany) at 10 μg/mL and the culture continued for 24 h. The cells were then harvested and their viability analyzed by flow cytometry.

2.3. Cell viability assay

Cell viability was assessed after staining with a commercial kit for discrimination of live and dead cells (Invitrogen, Carlsbad, CA, USA) by flow cytometry following the manufacturer's instruction with minor modifications. Briefly, DCs or PBMCs previously incubated with soluble cyt c were labelled with calcein-AM, and ethidium homodimer-1 or propidium iodide (PI, Sigma-Aldrich, Steinheim, Germany). Cells were considered alive when they were positive for calcein and dead (necrotic) when they were positive for ethidium or PI. Double negative cells were considered apoptotic. In some experiments calcein-labelled DCs were further stained with annexin V (BD Bioscience, Chicago, IL, USA) and PI in binding buffer or with anti-active caspase 3 (BD Bioscience, Chicago, IL, USA) after fixation and permeabilization using the commercial BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Bioscience, Chicago, IL, USA) according to manufactureŕs instructions. PBMCs were stained for CD19 (BD Bioscience, Chicago, IL, USA) for the discrimination of B lymphocytes and analyzed by flow cytometry as before.

2.4. ELISA for IL-12p70

The biologically active IL-12 (IL-12p70) was detected in supernatants of DCs cultures as described before (Baleeiro et al., 2013) using a commercial ELISA kit (Max Standard kit; BioLegend, San Diego, CA, USA) in 96-well microtiter plates (Nunc Maxisorp, Rochester, NY, USA) following the manufacturer's instructions.

2.5. Statistical analysis

Statistical analyses were done with Prism 2.01 for Windows (GraphPad Software, La Jolla, CA). Results from the experimental groups were compared by student's *t*-test and differences indicated as significant when $p < 0.05$.

3. Results and discussion

3.1. Export of antigen from endosomes into the cytosol in immature human DCs

Translocation of endocytosed proteins into the cytosol is deemed essential for cross-presentation in human and mouse DCs as was deduced from experiments with cyt c, which, when released as intact protein into the cytosol, it induces caspase-dependent apoptosis of the cells (Lin et al., 2008). In mice and humans, only a subset of DCs, dubbed cross-presenting DCs, were sensitive to cyt c-induced apoptosis, and only at very high concentration of cyt c (Lin et al., 2008; Farrand et al., 2009; Cockburn et al., 2011; Imai et al., 2011; Segura et al., 2013). We asked for the reason for the required high concentration of cyt c and the range of antigen-presenting cells, immature and mature DCs, B cells and monocytes, capable of translocating protein from endosomes into the cytosol. Immature monocyte-derived human DCs (iDCs) were generated from PBMC of healthy donors by 5 days culture in medium containing IL-4- and GM-CSF-. The cells were then incubated with horse cyt c for 24 h at concentrations ranging from 1 μg/mL to 10 mg/mL and their viability assessed after staining with calcein-AM and PI or ethidium homodimer 1. Cells negative for calcein and PI or ethidium were considered apoptotic. We found that after incubation with 10 mg/mL cyt c a high proportion of iDCs was apoptotic but not at lower concentrations (Fig. 1A, B). Previous reports had also shown apoptosis of DCs incubated with that concentration of cyt c (Lin et al., 2008; Farrand et al., 2009; Cockburn et al., 2011; Imai et al., 2011; Segura et al., 2013).

Following translocation into the cytosol, the proteins may be ubiquitinated and degraded by the proteasome whereby peptides for cross-presentation by MHC class I molecules are generated (Sijts et al., 2001), but cyt c would also lose its capacity to induce apoptosis. Houde et al. had shown that in DCs proteasomes are located in close proximity to endosomes/phagosomes (Houde et al., 2003) and concluded that this might facilitate processing of endocytosed Ags. We hypothesized that the induction of apoptosis by cyt c requires high concentrations of the protein because most in the cytosol is rapidly degraded by the proteasome. This is supported by experiments in which iDCs were incubated with cyt c in the presence of lactacystin, a proteasome inhibitor. Now, 10 μg/mL of cyt c was sufficient to efficiently cause apoptosis of iDCs (Fig. 1A, B). These results were confirmed by staining the cells with annexin V which detects phosphatidylserine, a lipid of the cell membrane that is flipped to the outer leaflet of apoptotic cells (Segawa and Nagata, 2015). In our experiments, calcein neg/PI neg iDCs were positive for annexin V (Fig. 1C). In addition, we could confirm that the proteasome inhibitor enhances the potency of cyt c to indeed induce cell death through apoptosis by staining for active caspase 3, a marker for cells undergoing apoptosis, which is generated through cleavage by other caspases or by autoproteolysis and plays a central role in the caspase cascades leading to apoptosis (Dai and Krantz, 1999). iDCs incubated with 10 μg/mL cyt c in the presence of lactacystin were