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RESEARCH HIGHLIGHT

Endocytosis by target cells: an essential means for perforin- and granzyme-mediated killing

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Despite the critical role of cell-mediated immunity in defense against intracellular pathogens, cancer immunosurveillance and development of autoimmunity, the precise mechanisms governing the killing of target cells by cytotoxic T cells (CTLs) and natural killer (NK) cells have remained poorly defined. In a study published in *Nature Immunology*, Thiery and colleagues have for the first time visualized the movement of the cytotoxic granule proteins perforin and granzymes from the killer cell to the target cell, challenging the textbook model of granule-mediated cytotoxicity and providing direct evidence for a multistep endocytosis-mediated mechanism for the entry of granzymes to the target cell's cytoplasm.[1]

Since the first observations of azurophilic granules in CTLs and NK cells and the identification of perforin in the 1980s,[2,3] a one-step model of cell-mediated killing has been widely accepted in spite of several inconsistencies. According to this model, exocytosis of cytoplasmic granules results in the release of perforin, which multimerizes to form large pores in the plasma membrane of the target cell, allowing entry of other granule proteins, including the serine proteases belonging to the granzyme family. Granzymes initiate apoptosis both through cleavage of caspase 3 and Bid and through caspase-independent mechanisms.[4]

Owing to difficulties in visualizing exocytosed perforin and granzyme, this model was developed largely on the basis of studies in which target cells were incubated with high concentrations of purified perforin; however, at concentrations high enough to form pores sufficiently large to allow the passage of granzymes, perforin induces necrosis rather than apoptosis, calling into question the physiological relevance of the classical model of perforin-mediated killing.[1,5] Indeed, at physiologically relevant concentrations, perforin has been shown to form much smaller pores in the plasma membrane, and these pores cannot accommodate granzymes.[5]

An alternative model, in which granzyme does not enter the target cell directly through plasma membrane pores, has thus been proposed. Instead, both perforin and granzyme are endocytosed by the target cell and subsequently released from an intracellular vesicle into the cytosol. Previous studies have provided support for this model by demonstrating receptor-independent endocytosis of granzymes by target cells;[4] however, the mechanisms mediating the putative endocytosis of granule contents and the subsequent release of endosomal cargo into the target cell's cytosol have remained unclear.

Using both biochemical methods and live-cell imaging of target cells upon treatment with sublytic concentrations of perforin or NK cell-mediated attack, Thiery *et al.* provide direct evidence for the following multi-step model (Figure 1):

1. Upon exocytosis of granule contents toward the immune synapse, perforin forms small pores in the target cell membrane, allowing the passage of Ca^{2+} but not larger molecules such as granzymes.
2. This Ca^{2+} influx induces the damaged membrane repair response, which involves the endocytosis of damaged portions of the membrane.
3. Rab5-dependent fusion of early endosomes results in the formation of large vesicles, termed gigantosomes, containing perforin and granzymes.
4. Perforin-mediated pore formation in gigantosome membranes inhibits endosomal acidification, preventing the destruction of granule contents in the gigantosome and allowing the release of granzymes into the target cell's cytosol.

The use of live-cell imaging techniques not only has allowed the visualization of gigantosome formation and the endocytic uptake of perforin and granzymes, but also has provided valuable information about the temporal and spatial dynamics of granzyme release into the cytoplasm. The authors found that gigantosomes formed within 5 min of treating target cells with a sublytic concentration of perforin and granzymes and that, 5 min later, perforin rapidly multimerized to form pores in the endosomal membrane. Importantly, western blotting of chemically crosslinked samples demonstrated that these multimers were sufficiently large to allow the passage of granzymes. Indeed, the release of granzymes from the gigantosomes was observed at the same time as pore formation, and granzymes had translocated to the nucleus within 20 min of perforin treatment.

Although the majority of the data presented by Thiery and colleagues were obtained by treating target cells with exogenous perforin and granzymes, these data were verified in an NK cell attack assay. In accordance with a previous report that target cells form large endosomes upon attack by CTLs (Keefe),[5] live-cell imaging demonstrated the presence of vesicles containing perforin and granzymes that appeared to be of a similar size to gigantosomes in B cells within 10 min of NK cell attack. As observed in perforin-treated target cells, granzymes were released from these vesicles within 20 min.

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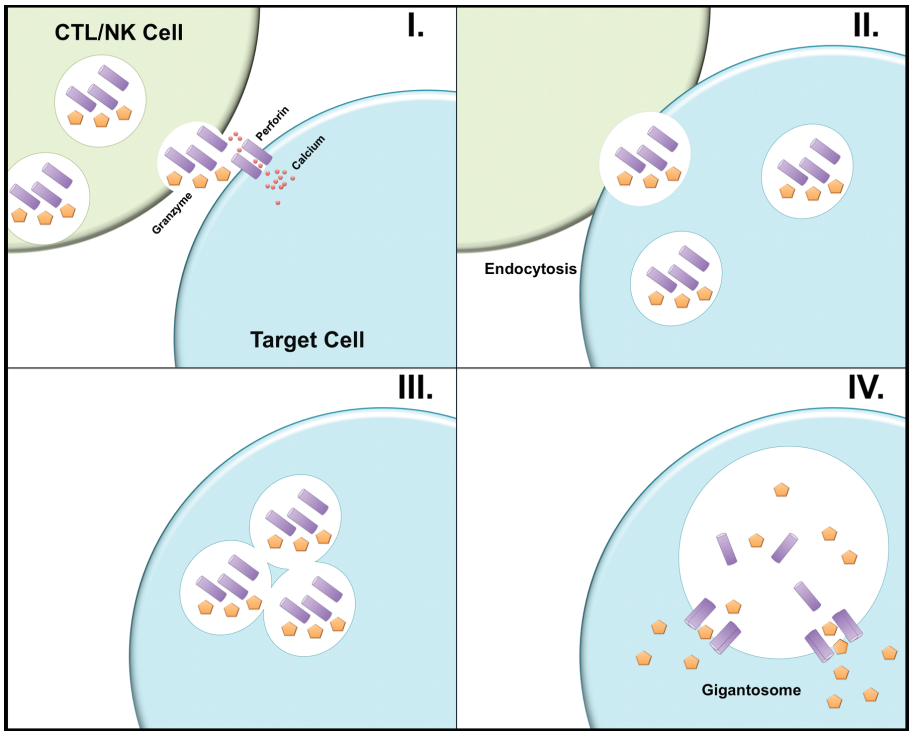

Figure 1 An endocytosis-based model for perforin- and granzyme-mediated killing of target cells. I.Perforin-mediated formation of small membrane pores allows the entry of Ca^{2+} into the target cell. II. Ca^{2+} influx triggers the damaged memberane response, resulting in endocytic uptake of CTL/NK cell memberanes and associated granule contents. III. Early endosomes fuse to form large vesicles termed gigantosomes. IV. Gigantosomes fail to acdify, and perforin-mediated formation of large pores in the gigantosome memberane allow release of granzyme into the cytosol.

Together, these data clearly demonstrate the entry of granzymes to the target cell's cytosol through an endocytic pathway. The essential role of endocytosis in granule-mediated killing is further demonstrated by the necrotic death of target cells upon the inhibition of endocytosis;[6] however, the contribution of the gigantosome itself to target cell death remains unclear. Not all target cells formed gigantosomes, and the inhibition of endosomal fusion did not affect target cell lysis. These findings suggest that although gigantosomes provide a convenient platform for observing the intracellular release ofgranule contents, the endosomal uptake of perforin and granzymes is sufficient for cell-mediated killing.

Importantly, these findings have therapeutic implications in infectious disease and cancer. Endocytic defects have been reported in multiple types of cancer, suggesting that, in addition to promoting tumor cell survival by preventing the downregulation of receptor tyrosine kinases, aberrant endocytosis may represent a mechanism by which tumor cells escape CTL killing.[7] Considering the key role of endocytosis in perforin-mediated killing demonstrated by Thiery *et al.*, enhancing endocytosis by target cells may become a new objective in vaccine design and cancer therapy.

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