

● A novel cytolytic molecule

It is a surprise to many immunologists that cytotoxic lymphocytes have very potent direct antimicrobial activity. It is accepted that lymphocytes play many roles in host defense to microbes. CD4 T cells, but also CD8 T cells and NK cells, have important immune regulatory roles. Cytotoxic lymphocytes have the ability to lyse host cells that harbor intracellular pathogens, and in the process of lysis of the host cells, the pathogens are often



Ling Ling Ma is a PhD student in immunology. She received her MD degree and M.Sc degree from Capital University of Medical Sciences in China and has performed research on immunopathogenesis of type 1 diabetes and T lymphocytes in microbial host defense. lma@ucalgary.ca

destroyed, or released into the extracellular environment where phagocytic cells can perform their effector function. However, cytotoxic lymphocytes also have direct antimicrobial activity. This antimicrobial activity requires cell contact and involves regulated exocytosis of granules in the cytoplasm of the effector cells. Granulysin, an antimicrobial protein, is a crucial element of this process.

Identification and expression of granulysin

The human granulysin cDNA was originally isolated by subtractive hybridization in a search for T-cell-specific molecules expressed "late" (3-5 days) after activation by either alloantigen or mitogen (1). Later, the gene was found to be constitutively expressed in NK cells (in contrast to activated expression in CD8 T cells) and localized to chromosome 2 (2).

Granulysin belongs to the saposin-like protein (SAPLIP) family. Proteins of this family all interact with lipids. Most closely related to granulysin are amoebapores A-C (3), and NK lysin (4). Amoebapores are present within the cytolytic granules of protozoa and are released directionally to kill bacteria. The other related member, NK lysin was isolated from pig intestines with antibacterial activity. All of the SAPLIP family members share a common structure, the SAPLIP domain, containing six conserved cysteine residues

Granulysin and Direct Lymphocyte-Mediated Antimicrobial and Antitumor Activity

Ling Ling Ma and Christopher H. Mody

Department of Microbiology and Infectious Disease, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, T2N 4N1, Canada

and several hydrophobic residues (5). Granulysin contains five-helix bundles and has a tyrosine instead of a cysteine at the first position.

Two forms of granulysin are produced by cytotoxic lymphocytes, which migrate with molecular mass of 15-kDa and 9-kDa. The 15-kDa protein is produced rapidly and has a shorter half-life, while the 9-kDa protein is produced more slowly and is relatively stable (6). The 9-kDa form is processed from the larger precursor 15-kDa form of granulysin (7), and purified 9-kDa protein kills a variety of microbial

such as *Cryptococcus neoformans* and *Candida albicans* (8).

Our studies focused on *C. neoformans*, a ubiquitous yeast and one of the most common life-threatening fungal infections in patients who have compromised cell-mediated immunity, including AIDS (10-12). *C. neoformans* can activate T lymphocytes via the *C. neoformans* mitogen (CnM) in its wall (13). The resultant activation is capable of killing the yeast. Previous studies have shown that cytotoxic T lymphocytes possess anticryptococcal activities, but these studies failed to identify the antimicrobial mechanism (14). To determine whether granulysin was the unidentified mechanism, we needed to inhibit granulysin specifically. However, blocking antibodies are ineffective because they do not gain access to the synapse between the cytotoxic lymphocyte and the target cell. Thus, it was necessary to apply a new technique to inhibit granulysin. For this purpose, we used RNA interference (RNAi), a newly developed



Christopher H. Mody MD is Professor, Departments of Internal Medicine and Microbiology and Infectious Diseases and a Senior Scholar of the Alberta Heritage Foundation for Medical Research at the University of Calgary. His research interests are in the area of microbial immunology and cell mediated host defense. cmody@ucalgary.ca

pathogens and tumor cells (8,9). Both the 15-kDa and 9-kDa granulysin are present in lower density granules of T cell and NK cells, however, only the 9-kDa granulysin is in the higher density granules, which are highly cytolytic (6).

Functions of granulysin

Purified granulysin is lytic against a broad range of microbes *in vitro*, including bacteria, fungi and parasites. It causes concentration-dependent growth inhibition of both Gram-positive and Gram-negative bacteria with greater than 1000-fold reduction in colony forming units for *Mycobacterium tuberculosis*, *Salmonella typhimurium*, *Listeria monocytogenes*, *E. coli*, and *Staphylococcus aureus*. Granulysin also kills parasites, such as *Leishmania major*, and fungi,

method of gene silencing (15-22). This method employs small interfering RNA (siRNA), which are 21 nucleotide RNA duplexes that had recently been applied to mammalian cell lines (23). Using siRNA, we obtained the evidence that the anticryptococcal activities of CD8 T cells were due to granulysin (24).

Mechanisms of action

The targets of granulysin include microbes and tumor cells (9). The lytic mechanism is distinct depending on the target. For microbial targets, osmotic lysis seems to be dominant. Granulysin interacts with the lipid component on the microbial surface, activating lipid-degrading enzymes, such as glucosylceramidase and sphingomyelinases (figure 1) (25). The highly charged granulysin molecule has

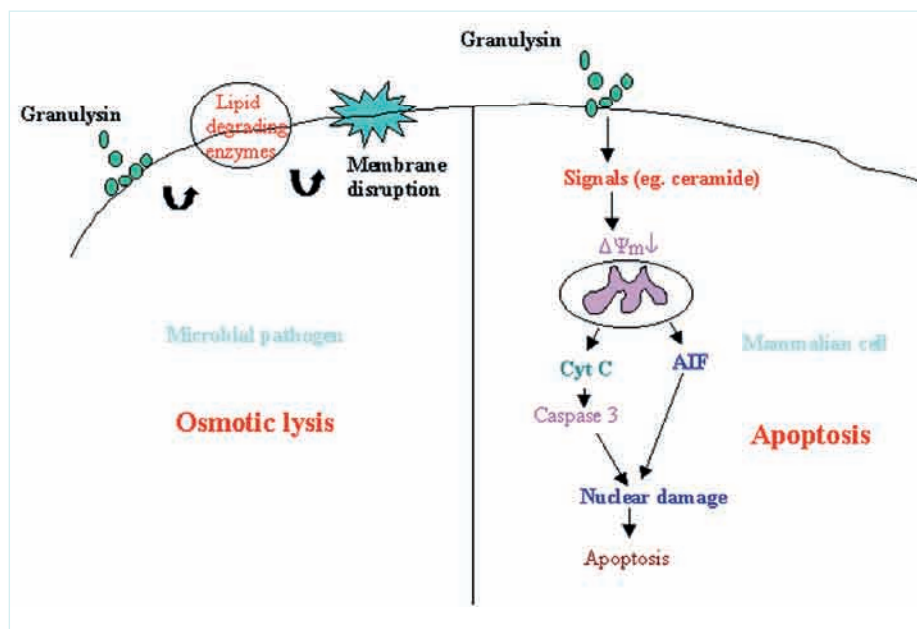


Figure 1: The mechanisms of granulysin function. Granulysin lyses the targets by osmotic lysis (in microbial targets) or induction of apoptosis (mammalian cell targets). Granulysin interacts with the membrane and activates the sphingomyelinase (SMase), results in membrane disruption and cell content leakage (left part). Granulysin also can induce target cell apoptosis mediated by mitochondria, cytochrome *c*, caspase 3, and apoptosis inducing factor (AIF) (right part).

also been proposed to induce pores in the membrane by a mechanism similar to electroporation (26-28). This leads to cell membrane disruption and the leakage of the cell contents, which causes cell death. *M. tuberculosis* has protruding lesions on the cell surface after exposure to granulysin (8), and an increase in membrane permeability of the *M. tuberculosis* and *E. coli* results in osmotic lysis (26).

By contrast, granulysin induces apoptosis in tumor cells. It causes an increase in mitochondrial membrane permeability and the release of cytochrome *c* and apoptosis inducing factor (AIF) (29,30). Despite causing cytochrome *c* release, granulysin-induced mitochondrial damage dose not cause procaspase 9 activation via the classical apoptosome, but it still manages to activate caspase 3. Caspase 3 and AIF induce nuclear fragmentation and condensation, resulting in apoptosis (29,30) (Figure 1).

● Perforin and granulysin

Perforin, a calcium-dependent pore-forming protein, is an important component of granule exocytosis and cell-mediated cytotoxicity. Perforin entry into the tumor target cell membrane creates a signal for the target cell to repair the damage by endocytosing the perforin and surrounding plasma membrane. Granzymes (distinct from granulysin) in the vicinity of the lesion are also endocytosed and ultimately delivered

to the target cell cytoplasm and nucleus, where they deliver proapoptotic signals (31). Although the mechanisms by which perforin contributes to granulysin mediated cytotoxicity are not clear, it is apparent that perforin is involved in some forms of granulysin-mediated cytotoxicity.

When microbes are extracellular, granulysin functions independently of perforin. Killing of *C. neoformans* is an example of this. When activated CD8 T cells came into contact with *C. neoformans*, *C. neoformans* was killed in the presence of perforin inhibitors, suggesting that granulysin-mediated anticryptococcal activity is independent of perforin (24). This is supported by the observation that recombinant granulysin killed more than 90% of extracellular *M. tuberculosis* in co-culture (8). By contrast, killing intracellular *M. tuberculosis* by granulysin depends upon perforin. The combination of recombinant granulysin and purified perforin was highly effective at killing intracellular *M. tuberculosis* (8). It may be that perforin facilitates the entry of granulysin into the host cell where it effects the intracellular microbe. Alternately, the host cell might be lysed by a perforin-dependent mechanism, releasing the microbe into the extracellular milieu where it would be susceptible to granulysin-mediated killing.

● Granulysin and cytokines

Cytokines such as IL-15 are critical ele-

ments in the control of cytotoxic activity. IL-15 is a member of the four-helix bundle cytokine family with growth factor activity for T cells and NK cells (32,33). It is produced by many cell types including monocytes, macrophages (34,35) and dendritic cells (36), but not by T cells (37). IL-15 also promotes the survival and proliferation of memory lymphocytes (37-39), and induces NK cells to be cytolytic effector cells (40).

Previously, it had been established that IL-15 is required for lymphocyte-mediated anticryptococcal activity (41). When we found that the anticryptococcal activities of CD8 T cells were mediated by granulysin, we considered whether IL-15 might regulate the levels of this cytolytic molecule. We found that IL-15 could directly upregulate the active form of granulysin in primary human CD8 T cells, even in the absence of CD4 T cells and accessory cells (24). Thus, IL-15 was sufficient to upregulate granulysin expression. Further, CD8 T cells isolated from peripheral blood mononuclear cells that had been stimulated with CnM expressed both forms of granulysin. However, when CD8 T cells were isolated from CnM stimulated PBMC in the presence of the anti-IL-15 antibody, granulysin expression was eliminated (24). Thus, in addition to being sufficient, IL-15 was also necessary for the upregulation of granulysin.

● CD4 T cells and Granulysin

CD4 T helper cells play an essential role in the activation of CD8 T cells. CD4 T cells secrete T cell growth factors such as IL-2, which activate CD8 T cells (42). Additionally, CD4 T cells signal accessory cells, such as dendritic cells, monocytes and macrophages, to express important cytokines such as IL-1, IL-15, or TNF α (41-44), or surface expressed ligands, such as CD40, LFA-1 or ICAM-1 (47,48), so that CD8 T cells can be activated (49). Our work demonstrated that CD4 T cells were required for CD8 T cell proliferation in response to *C. neoformans*, but this effect was not due to IL-2 alone (50). Recently, we demonstrated that CD4 T cells were required for the CD8 T cells to produce granulysin and to become cytotoxic cells with anticryptococcal activity (24).

Thus, both CD4 T cells and IL-15 were required to upregulate granulysin expression in CD8 T cells. There are at least two mechanisms that might explain the observation that both CD4 T cells and IL-15 are required. First, *C. neoformans*

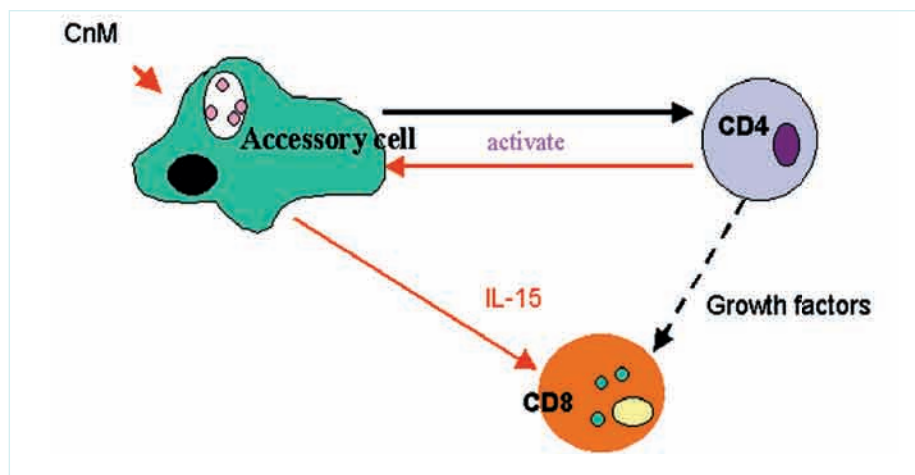


Figure 2: Model of CD4 T cell, accessory cell, and IL-15 involvement in regulation of granulysin expression in CD8 T cells. CnM is presented by accessory cells to CD4 T cells (solid black line). While CD4 T cells could produce growth factors that activate CD8 T cells to make them antifungal (dashed black line), IL-15 is a sufficient factor for upregulation of granulysin expression, which makes it more likely that CD4 T cells activate accessory cells, which produce IL-15 that results in upregulation of granulysin in CD8 T cells.

could stimulate accessory cells to express IL-15. Presentation of CnM by accessory cells, in combination with IL-15 would signal CD4 T cells to secrete T cell growth factors that would stimulate granulysin expression in CD8 T cells. This would be consistent with the observation that IL-15 is necessary for granulysin expression, and that CD4 T cells and accessory cells are required for granulysin expression and the antifungal effect in CD8 T cells (figure 2, black lines). Alternately, *C. neoformans* could stimulate CD4 T cells, which would stimulate the accessory cells to express IL-15, which would then activate the CD8 T cells to express granulysin and exhibit antifungal activity. However, IL-15 can replace the requirements for both accessory cells and CD4 T cells, and CD4 T cells do not release IL-15 (37). This is most consistent with the second model in which CD4 T cells are activated, they stimulate accessory cells of the myeloid lineage to produce IL-15, which then activates CD8 T cells for antifungal activity (figure 2 red lines).

● Summary

Granulysin is a newly described lytic molecule expressed by cytotoxic T-lymphocytes and NK cells. It possesses a broad spectrum of antimicrobial activities and tumoricidal activities. Granulysin lyses microbial targets via osmotic lysis and induces apoptosis of some tumor cells. Granulysin is necessary for CD8 T cell-mediated antimicrobial activity. The granulysin-mediated anticytotoxic activity is perforin independent. In response to CnM, CD4 T cells are required to activate accessory cells that secrete IL-15, which upregu-

lates granulysin expression and enhances the anticytotoxic activity.

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