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A novel role for granzymes in anti-tumor immunity

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Abbreviations: CL, cytotoxic lymphocytes; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GrAB, granzyme A and B; PS, phosphatidylserine

The cytotoxic properties of granzymes are well established, though recent publications suggest additional roles for granzymes in immunity. We demonstrated that granzymes can act as regulators of cross-presentation by dendritic cells by inducing critical "*eat-me*" signals on the dying tumor cell, resulting in efficient phagocytosis of cell-associated tumor antigen.

One of the key mechanisms used by cells of the immune system to eliminate cancer cells is their recognition by cytotoxic lymphocytes (CL), which comprise natural killer cells as part of the innate, and cytotoxic T lymphocytes (CTL) within the adaptive immune system. Upon recognition, CL kill tumor cells by either engaging death receptors or by granule exocytosis, whereby proteases are released into the immune synapse, cross the target cell membrane through perforin pores and finally induce apoptosis by cleaving their various substrates. The key death-inducing effector molecules are thought to be granzyme A (GrA) and granzyme B (GrB).[1] The direct cytotoxicity of granzymes is typically regarded as an important anti-tumor function in itself, however the ultimate fate of tumor cells killed via granzymes has never, until recently, been explored in detail. In our work, we demonstrated a critical role for granzymes in anti-tumor immunity by showing that they induce pro-phagocytic signals on the dying tumor cell.[2]

Tumor cells killed by GrAB-sufficient CL show morphological signs of apoptosis such as rounding, blebbing, lifting from the substrate and chromatin condensation.[3] Furthermore, time-lapse microscopy confirmed the early exposure of phosphatidylserine (PS) followed some time later by loss of membrane integrity, indicating secondary necrosis. In contrast, CL deficient for GrAB showed morphological characteristics of apoptosis but lacked early PS exposure until the cell membrane became permeable for a DNA intercalating dye.[3] As PS is regarded as one of the most critical signal for uptake of apoptotic cells, we explored the functional significance of tumor cell death in the absence or presence of GrAB. We used GrAB-sufficient or -deficient CTL to kill antigen-specific tumor cells carrying the model antigen ovalbumin (OVA) and co-cultured dying tumor cells with dendritic cells (DC).[2] We found that CD8α$^+$ DC showed a marked reduction in OVA cross-presentation in vitro and in vivo when exposed to tumor cells killed in the absence of GrAB. By contrast, the absence or presence of GrAB had no effect on the expression of activation makers and secretion of cytokines by the CD8α$^+$ DC. Nevertheless, the MHC class I molecules of CD8α$^+$ DC were significantly occupied by the OVA peptide SIINFEKL only when the DC were co-cultured with tumor cells killed by GrAB-sufficient CTL. Finally, we were able to pin down the impaired cross-presentation by DC to a marked reduction of phagocytosis of the dying tumor cells. By using Annexin V to block PS, we found that PS per se is not critical for tumor cell uptake by DC, confirming previous studies using UV-irradiated tumor cells.[4] From these experiments, we further concluded that granzymes induce other pro-phagocytic "*eat-me*" signals apart from PS on the outer membrane of dying tumor cells (**Fig. 1**).[2]

Our findings have implications for various aspects of the immune response against cancerous cells. First and most obviously, we showed that granule-mediated cell death induces an immunogenic form of cell death leading subsequently to greatly amplified tumor antigen-specific effector responses. Immunogenic cell death has been described for several cell death inducing agents such as chemotherapeutic drugs that can act either by facilitating engulfment by antigen-presenting cells, or by releasing activating signals such as ATP or HMGB-1,[5] resulting in anti-tumor immunity. Furthermore, once an adaptive immune response is induced to one particular tumor antigen, the early phase of CTL killing may result in epitope spreading

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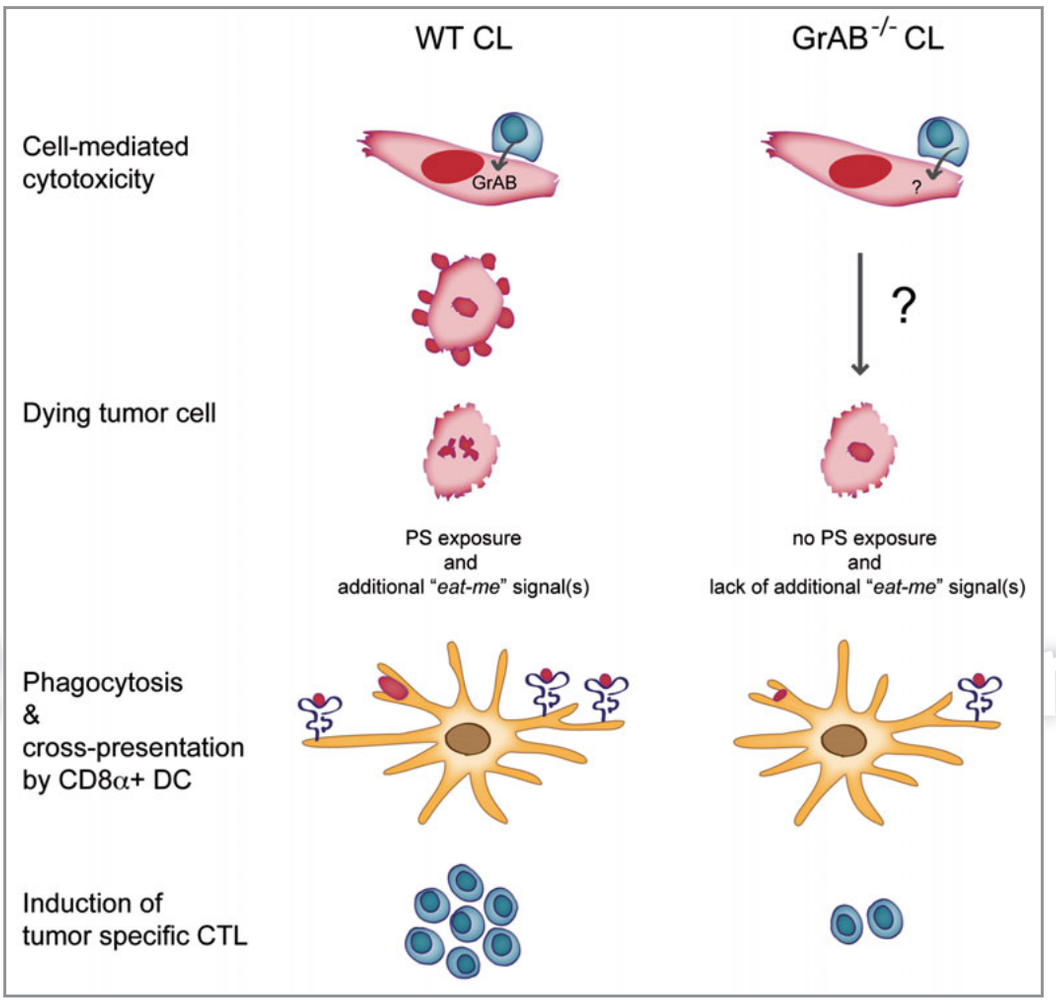

Figure 1. Cell-mediated killing of tumor cells by wild-type cytotoxic lymphocytes (WT CL) via granzymes A and B (GrAB) results in the exposure of yet unknown pro-phagocytic "*eat-me*" signals in addition to phosphatidlyserine exposure (PS). The CD8α$^+$ DC engulf dying tumor cells and cross-present tumor antigen to naïve cytotoxic T lymphocytes (CTL), inducing in turn an adaptive immune response against the tumor. However, tumor cell death in the absence of GrAB (GrAB$^{-/-}$ CL) lacks PS exposure and other pro-phagocytic "eat-me" signals leading to reduced phagocytosis, cross-presentation and subsequent induction of tumor specific CTL.

and consequently, a broadened adaptive T cell response to additional tumor-associated antigens. In human cancer patients, various therapies such as vaccination or adoptive T cell therapy should benefit from epitope spreading and therefore counteract tumor immune escape caused by the selective loss of the dominant tumor antigen.[6] However, similar studies with human tumor cells killed exclusively via granule exocytosis are needed to confirm our results in a human setting, as mouse and human GrB show subtle but important differences in substrate preference.[7]

Futhermore, it is well established that tumor cells can become resistant to receptor mediated apoptosis via TNFα, FasL or TRAIL by downregulating extracellular receptors or by upregulating cytoplasmatic expression of inhibitors such as dominant-negative Fas-associated death domain (FADD) or intracellular FADD-like inhibitory protein (FLIP).[8] In such instances, the elimination of tumor cells relies solely on the perforin/granzyme pathway. Under these conditions we postulate that the lack of expression of GrAB by CL or the additional expression of intrinsic granzyme inhibitors (serpins) by the tumor would result in markedly impaired phagocytosis, and therefore, reduced cross-presentation of tumor antigen leading to a poor anti-tumor immunity.

Our study contributed a novel function for granzymes to the constantly growing body of evidence that granzymes have many more functions in addition to their well-established role in direct, perforin-dependent cytotoxicity. Recently, GrA was found to induce pro-inflammatory cytokine release in myeloid cells through a perforin-dependent mechanism that also extended to granzyme M in a TLR-4 dependent endotoxic shock model.[9,10] Our study revealed that granzyme-mediated cell death plays a significant role critical for the induction of immunity to tumor antigen, by regulating the phagocytic uptake of dying tumor cells. However, the specific nature of the phagocytic signal remains to be elucidated; its identification will also have significant impact on studies in dendritic cell biology.

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