
A Molecular View of Cytotoxic T Lymphocyte Induced Killing

R. Chris Bleackley

Department of Biochemistry, University of Alberta, Edmonton

Cytotoxic T lymphocytes (CT) and natural killer (NK) cells serve to protect us against viruses and tumours, and are key players in rejection of transplants and autoimmune disorders. They seek out and bind to targets, then deliver signals that result in the ultimate demise of the recognized cell. In this article I will summarize a number of the contributions that my laboratory has made to understanding how these effectors bring about target cell destruction. Other researchers have made numerous and significant contributions and we have recently published more comprehensive reviews (1,2,3).

Granzyme B – A natural born killer

The major efforts of research in my laboratory have been directed at understanding the nature of these death signals at the molecular level. Most of the early work in this area focused on electron-dense granules that exist in the cytoplasm of cytolytic effector cells. It was believed that they contained the lytic molecules that mediated cell death. When the CTL bind to a target the granules polarize to the contact surface with the target and thus the cytolytic effector(s) is delivered in a directed fashion.

One of the first proteins to be isolated from the granules was perforin. Biochemically purified perforin can readily bring about lysis of red blood cells and it was envisaged that this membraneolytic activity was solely responsible for target cell death. However, it was soon realized that although perforin could mediate membrane damage, it was unable to initiate DNA fragmentation. The latter being a hallmark of death when a target cell is treated with intact CTL (4).

At this time my laboratory had isolated a gene that encoded a serine protease that is now known as

granzyme B (5). Expression of the protease correlates with the killing activity of CTL and the protein is found in granules (6,7). In collaboration with Michael James we predicted that this proteolytic enzyme would cleave substrates at aspartate residues (8). This is considered to be a very unusual specificity for a serine protease and was a critically important idea in our understanding of how granzyme B might bring about death. However, another important result came from Arnold Greenberg (Manitoba), who demonstrated that perforin supplemented with granzyme B (called fragmentin by Arnie) could recapitulate both membrane damage and DNA fragmentation. The model of CTL-mediated lysis was then modified to include granzyme B as a major player. The granules now delivered both perforin and granzyme, and then perforin created the channel through which the protease fused to gain access to the target cell cytoplasm. Through the cleavage of substrates, granzyme B initiated a program of cell death that includes membrane disruption and DNA fragmentation that we now refer to as apoptosis (9).

Obviously a critical question was the identity of these substrates. Bob Horwitz (MIT) had identified a key protein necessary for apoptosis in nematodes which he named ced3. This is a cysteine protease that requires activation through proteolytic cleavage at an aspartate residue. This was exactly the specificity we had predicted for grB (8,10). With considerable help from Don Nicholson (Merck Frosst) we were able to prove that the mammalian homologue of ced3, caspase3, was indeed a critical substrate for grB (11). To prove this in vivo we generated CTL from wild type and grB knock out mice and used these to kill targets. With the wild type CTL we saw very nice activation of caspase 3 but with the knock outs this was completely suppressed (12).

The story seemed complete with grB acting on caspase 3 to initiate apoptosis as depicted in the left side of Figure 1. As usual experimental results seemed to get in the way of this nice model. When we used an inhibitor of caspase 3, DEDV-CHO, DNA fragmentation was nicely suppressed but other features of death, notably membrane damage, were not. The model was then modified to account for these findings (12,13). The activation of caspase resulted in DNA fragmentation but other grB-substrates needed to be cleaved in order to bring about membrane damage. The model at this time is given in Figure 1 where we have grB cleavage of caspase 3 leading to apoptosis but now with another arm to the pathway. Again we were left with the critical question regarding the identity of these proteins.

Mitochondrial involvement in death

A very important clue came from experiments that we were conducting on Bcl-transfected cells in order to study Fas-mediated killing. To our surprise numerous independent clones of transfected cells were not only resistant to Fas killing but also to both membrane lysis and DNA fragmentation mediated by grB. A major site of action of the anti-apoptotic Bcl2 is mitochondria. We therefore tested whether mitochondrial disruption was occurring in the granzyme pathway. Cells were labeled with a fluorescent dye that accumulates in healthy mitochondria but is lost when the organelle is compromised. When these cells were used as targets for either grB or CTL there was rapid loss of the dye. Interestingly this occurred in the presence of caspase inhibitors, thus arguing for the involvement of a direct effect of grB in the pathway to mitochondrial disruption (14).

The mechanism for Fas mediated killing involves caspase 8 which cleaves a proapoptotic member of the Bcl2 family, Bid. This cleavage occurs at aspartate-59, but close by is another sequence that looked like a good grB substrate. Using a variety of *in vitro* and cellular assays we were able to prove that Bid is indeed a good substrate for grB and that its activation is critical in mitochondrial effects in association with Bax (15,16).

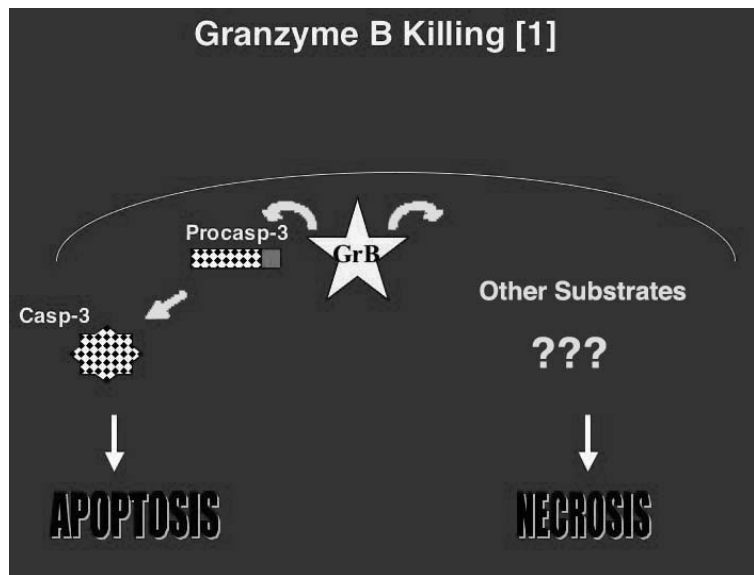


Figure 1 Granzyme B Killing [1]. The simplest view of killing suggested passage of grB through a perforin channel and then cleavage of caspase 3 to bring about apoptosis. Further experiment suggested that some aspects of cell death were caspase independent and therefore cleavage of alternate substrates was proposed.

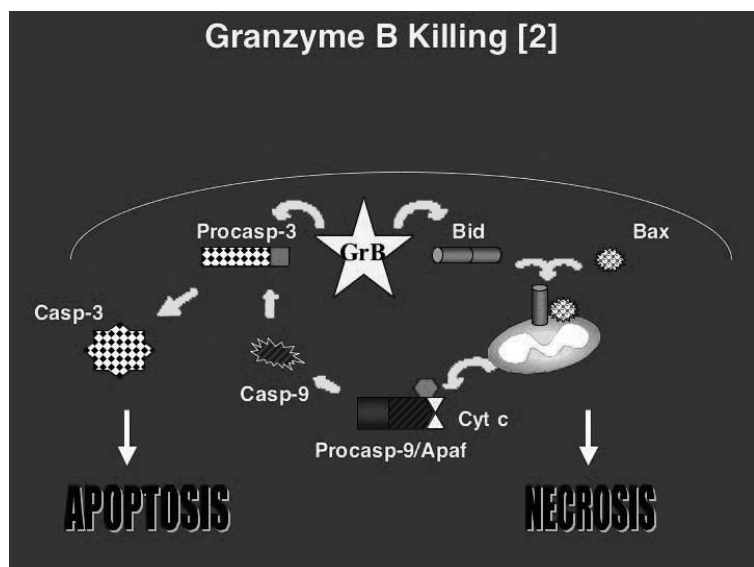


Figure 2 Granzyme B Killing [2]. Internalized grB cleaves and activates caspase 3 to bring about apoptosis. Further cleavage of Bid causes translocation with Bax to the mitochondria. This can lead to release of cytochrome c into the cytoplasm which stimulates formation of the apoptosome. As a result caspase 9 is activated which can amplify the cleavage of caspase 3. In addition disruption of mitochondrial functions can lead to necrotic death.

Granzyme B can thus initiate cell death through at least two pathways. The first involves activation of caspase 3 and secondly via mitochondrial disruption after cleavage of Bid (17,18). This latter route would lead to loss of mitochondrial functions and ultimately necrotic death. However, cleaved

bid also brings about translocation of cytochrome c into the cytosol which is critical in formation of the apoptosome and activation of caspase 9. Our model (Figure 2) evolved to suggest that the mitochondrial pathway could result in activation of caspase 9 (and caspase 3) and necrosis through disruption of mitochondria function. We tested whether caspase 9 was essential for grB-mediated killing by transfection of cells with a dominant negative version of the enzyme. While the inhibitory version of caspase 9 could efficiently abrogate Fas-mediated death (known to go through caspase 9), it only minimally suppressed grB-mediated DNA fragmentation. Thus the mitochondrial involvement in the granzyme pathway did not appear to require caspase 9 (19).

The Safety Brake Model

In reviewing our Bcl2 inhibition results we noted that caspase 3 cleavage was blocked at p20 when the transfected cells were treated with granzyme B. This was identical to the Western blot profile we observed when cells were killed in the presence of a caspase 3 inhibitor. In order to understand this result it needs to be appreciated that caspase 3 activation is a two stem process. The first involves the direct action of grB on the precursor p32 molecule to produce p20 and p10 fragments. Then the

low level of activity of the enzyme is enough to promote self catalytic proteolysis of p20 to p19. The resulting p19/p10 heterodimer is the fully active form of caspase 3. Thus Bcl2 is acting to inhibit, indirectly, the self catalytic step.

The activity of caspases can be suppressed in the presence of inhibitors of apoptosis proteins (IAPs) with Bob Korneluk and Peter Liston (CHEO). We therefore asked whether the IAP family member XIAP could block grB-mediated caspase 3 activation *in vitro*. Indeed when we added XIAP to grB and caspase 3 we saw a build up of p20. In addition when a cell line was infected with an adenovirus encoding XIAP we observed almost complete blockade of the DNA fragmentation normally seen with grB (19).

Many cells express IAPs but caspases can still be activated. This is achieved by the action of proteins (e.g. SMAC/Diablo/Omi) that efficiently bind to IAPs and thus overcome the IAP-mediated inhibition of caspase activity. In our *in vitro* assay with grB, caspase 3 and XIAP, a SMAC peptide was indeed able to overcome the p20 blockage and appearance of p19 was seen.

Taking this together we have proposed a "Safety Brake Model of Killing" (Figure 3) in which grB cleavage of caspase 3 is analogous to pressing the gas pedal. However, the hand brake (IAP) is on so apoptosis does not proceed. Only after bid cleavage and SMAC release from the mitochondria can the death pathway proceed (2,19).

Our model works well for the reductionist view of killing with purified grB but does not explain the situation with intact CTL. While Bcl2 can efficiently block grB-mediated death it is not particularly effective when targets are treated with intact effector cells. This result is mirrored in the activation of caspase 3, where in the purified system activation is inhibited by Bcl but with cells it is not. We believed that the simplest explanation was that CTL killing involves whole granules and these likely contained other lytic effectors. However, when intact granules were used in killing or caspase 3 activation assays, Bcl2 transfected targets were refractory. Our current hypothesis is that

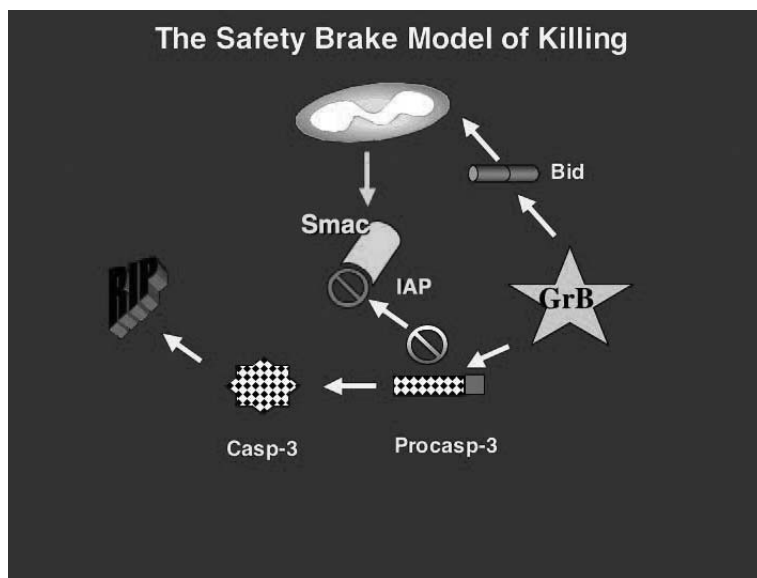


Figure 3 The Safety Brake Model of Killing. Granzyme B cleaves caspase 3 but activation of caspase 3 is blocked by IAPs. In order to overcome this inhibition Bid cleavage results in Smac release from the mitochondria and binding of SMAC to IAP. Only then is full caspase 3 activity revealed.

cell-cell contact between effector and target is important. Perhaps a signal is transduced into the target that inactivated the Bcl2 inhibitory activity. The molecular basis of this mechanism is currently under investigation.

The hole story is not the whole story

Returning to one of the early models of killing, it was believed that perforin acted to create a channel, in the target cell membrane, through which granzyme could pass. There is very little evidence, however, for this model and in fact perforin has never been shown to allow passage of a 30 kDa protein (the approximate mass of grB). Indeed we observed that grB, with a fluorescent tag, could be taken up into cells independently of perforin (20). On the basis of these observations it was proposed that a receptor existed for the proteinase, and we provided evidence that the mannose-6-phosphate receptor could act to internalize grB (21).

In early studies we demonstrated that uptake was dependent on the presence of the receptor and this could be inhibited by mannose-6-phosphate. However, it became apparent that some cell types did not appear to require the receptor as M6P inhibition was minimal when purified grB was used. The strange result was that these same cells were sensitive to M6P inhibition when intact granules were used as the killing signal. It turns out that grB exists in a very high molecular weight form in granules or in freshly isolated degranulate material. This complex contains minimally granzymes, perforin and proteoglycan and is capable of killing targets. As depicted in Figure 4 we can envisage at least three possibilities. Perforin could polymerize to create a channel for granzyme to pass which is shown on the left side. On the right hand side the whole complex binds to the receptor and is internalized with perforin mediating release from endosomes. The alternative (shown in the middle) has grB binding nonspecifically to the membrane with perforin stimulating same kind of repair mediated entry. Experimentally killing with the degranulate material can be inhibited through expression of a dominant negative dynamin protein with the target cell. In contrast death induced by purified grB

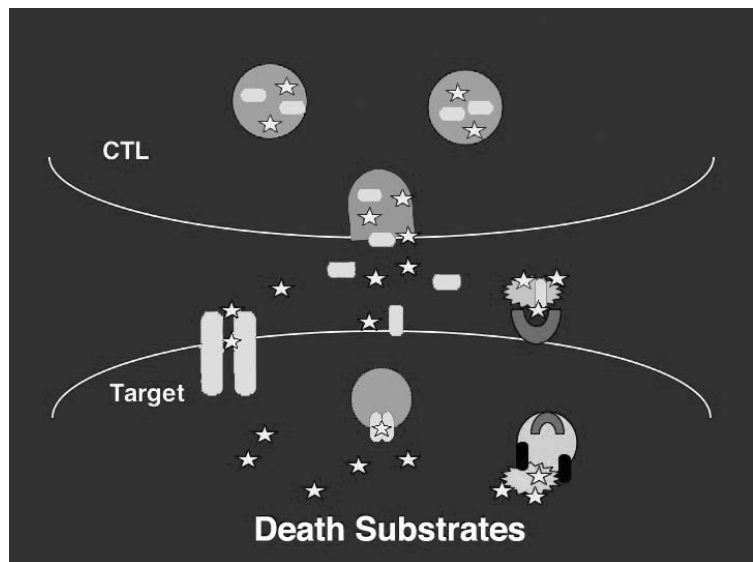


Figure 4 Models of Granzyme Entry. Granzyme B is present in the CTL-granule as a complex with perforin and proteoglycan. This complex is stable after degranulation and thus may bind intact to the receptor (right hand side). After internalization perforin will mediate release of grB into the cytosol. Alternately if the complex dissociates grB could either pass through a perforin channel (left hand side) or be taken up by a perforin-induced repair mediated pathway (middle).

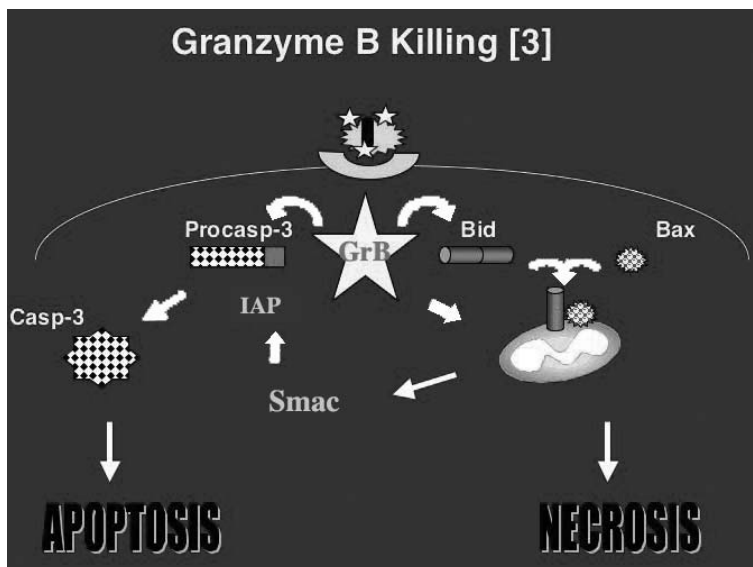


Figure 5 Granzyme B Killing [3]. In this current model grB is taken up as a complex. Once released in the cytosol it brings about the cleavage of caspase 3, disruption of mitochondrial functions and release of regulators of apoptosis.

is not sensitive to the inhibitor (22). We interpret this to mean that killing with the complex depends on receptor-mediated endocytosis, a process known to depend on dynamin. Obviously the extent of the inhibition will depend on the amount of free versus complexed grB that is present within the immunologic synapse (Figure 4).

Interestingly when targets that express the DN were treated with intact CTL a significant inhibition of death was observed (22).

We believe this pathway could be quite important as the M6PR, under its other guise as the insulin like growth factor 2 receptor, has been characterized as a tumour suppressor in a wide variety of human tumours. Thus lack of expression of the receptor on the cell surface could be a strategy to avoid the lethal consequences of recognition by a CTL or NK cell.

A Work in Progress

Over the years our model of killing has evolved to take into account new and often unexpected results. Our almost current view of granzyme B-mediated death is shown in Figure 5. Once grB is internalized, via interaction with a receptor, it can bring about the demise of the cell via numerous pathways including cleavage of caspases, disruption of mitochondrial function, and release of key apoptosis regulatory proteins. However it is also known that the proteinase cleaves other substrates that may also contribute to the death of the unfortunate target. This may appear to be overkill but may be not, when you consider that the function of these cytolytic effectors is to combat viruses that have a variety of tricks to preserve themselves. In addition most of our experimental data is generated with *in vitro* systems and it is clear that the situation with intact CTL and *in vivo* responses will likely be more complex. As we strive to move to more physiologically relevant experiments it is likely that additional pathways will be revealed. We believe that understanding these pathways to death and strategies for survival will provide vital information for the development of immunotherapeutics.

Acknowledgements

I have been extremely fortunate over the years to have worked with numerous extremely talented and thoughtful students, fellows, technologists and collaborators. In addition, through most of this time, my secretary Mae Wylie has transcribed and corrected my rough copy. This work would not have been performed without the generous support

of AHFMR, NCIC, MRC, CIHR and HHMI. Finally, thanks to CSBMCB and Roche for bestowing the honour of this award.

Bibliography

1. Barry, M. & R.C. Bleackley. Cytotoxic T Lymphocytes: All roads lead to death. *Nature Reviews Immunology* 2:401-409 (2002).
2. Roberts, D.L., I.S. Goping & R.C. Bleackley. Mitochondria at the heart of the cytotoxic attack. *Biochem. Biophys. Res. Comm.* 304:513-518 (2003).
3. Lord, S.J., R.V. Rajotte, G.S. Korbitt & R.C. Bleackley. Granzyme B: a natural born killer. *Immunological Reviews* 193:31-38 (2003).
4. Helgason, C.D., L. Shi, A.H. Greenberg, Y. Shi, P. Bromley, T.G. Cotter, D.R. Green & R.C. Bleackley. DNA fragmentation induced by cytotoxic T lymphocytes can result in target cell death. *Exper. Cell Res.* 206:302-310 (1993).
5. Lobe, C.G., B. Finlay, W. Paranchych, V.H. Paetkau & R.C. Bleackley. Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science* 232:858-861 (1986).
6. Lobe, C.G., C. Havele & R.C. Bleackley. Cloning of two genes which are specifically expressed in activated cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 83:1448-1452 (1986).
7. Redmond, M.J., M. Letellier, J.M.R. Parker, C. Lobe, C. Havele, V. Paetkau & R.C. Bleackley. A serine protease (CCP1) is sequestered in the cytoplasmic granules of cytotoxic T lymphocytes. *J. Immunol.* 139:3184-3188 (1987).
8. Murphy, M.E.P., J. Moulton, R.C. Bleackley, I.L. Weissman & M.N.G. James. Comparative molecular model building of two serine proteinases from cytotoxic T lymphocytes. *Proteins: Structure, Function and Genetics* 4:190-204 (1988).
9. Atkinson, E.A. & R.C. Bleackley. Mechanisms of lysis by cytotoxic T cells. Invited review in *Critical Reviews in Immunology* 15:359-384 (1995).

-
10. Caputo, A., M.N.G. James, J.C. Powers, D. Hudig & R.C. Bleackley. Conversion of the substrate specificity of mouse proteinase granzyme B. *Nature Structural Biology* 1:364-367 (1994).
 11. Darmon, A.J., D.W. Nicholson & R.C. Bleackley. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. *Nature* 377:446-448 (1995).
 12. Darmon, A.J., T.J. Ley, D.W. Nicholson & R.C. Bleackley. Cleavage of CPP32 by granzyme B represents a nonredundant role for granzyme B in the induction of target cell DNA fragmentation. *J. Biol. Chem.* 271:21709-21712 (1996).
 13. Darmon, A. & R.C. Bleackley. Proteases and cell-mediated cytotoxicity. *Crit. Rev. Immunol.* 18:255-273 (1998).
 14. Heibein, J.A., M. Barry, B. Motyka & R.C. Bleackley. Granzyme B-induced loss of mitochondrial inner membrane potential ($\Delta\psi_m$) and cytochrome c release are caspase-independent. *J. Immunol.* 163:4683-4693 (1999).
 15. Barry, M., J.A. Heibein, M.J. Pinkoski, S-F. Lee, R.W. Moyer, D.R. Green & R.C. Bleackley. Granzyme B short circuits the need for caspase 8 activity during granule-mediated CTL killing by directly cleaving Bid. *Molec. Cell Biol.* 20:3781-3794 (2000).
 16. Heibein, J.A., I.S. Goping, M. Barry, M.J. Pinkoski, G.C. Shore, D.R. Green & R.C. Bleackley. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and bax. *J. Exp. Med.* 192:1391-1401 (2000).
 17. Bleackley, R.C. & J.A. Heibein. Enzymatic control of apoptosis. *Nat. Prod. Rep.* 18:431-440 (2001).
 18. Pinkoski, M.J., N.J. Waterhouse, J.A. Heibein, B.B. Wolf, T. Kuwana, J.C. Goldstein, D.D. Newmeyer, R.C. Bleackley & D.R. Green. Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway. *J. Biol. Chem.* 276:12060-12067 (2001).
 19. Goping, I.S., M. Barry, P. Liston, T. Sawchuk, G. Constantinescu, K. Michalak, I. Shostak, D.L. Roberts, A.M. Hunter, R. Korneluk & R.C. Bleackley. Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* 18:355-365 (2003).
 20. Pinkoski, M.J., M. Hobman, J.A. Heibein, K. Tomaselli, F. Li, P. Seth, C.J. Froelich & R.C. Bleackley. Entry and trafficking of granzyme B in target cells during granzyme B-perforin mediated apoptosis. *Blood* 92:1044-1054 (1998).
 21. Motyka, B., G. Korbitt, M.J. Pinkoski, J.A. Heibein, M. Barry, A. Caputo, M. Hobman, T. Sawchuk, I. Shostak, C.F.B. Holmes, J. Gaudie & R.C. Bleackley. Mannose 6-phosphate/insulin-like growth factor II receptor is a death receptor for granzyme B during cytotoxic T cell-induced apoptosis. *Cell* 103:491-500 (2000).
 22. Veugelers, K., B. Motyka, C. Frantz, I. Shostak, T. Sawchuk & R.C. Bleackley. The granzyme B-serglycin complex from cytotoxic granules requires dynamin for endocytosis. *Blood* 103:3845-3853 (2004).
-