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Review

Granzymes, cytotoxic granules and cell death: the early work of Dr. Jurg Tschopp

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Within the powerful legacy left by Jurg Tschopp, we should not forget his early work that helped to elucidate the molecular pathways responsible for the clearance of virus-infected and transformed cells by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. Jurg's skilful biochemical approach formed a firm platform upon which the work of so many other biochemists, cell biologists and immunologists would come to rely. Jurg coined the shorthand term 'granzyme' to denote the individual members of a family of serine proteases sequestered in and secreted from the cytotoxic granules of CTL/NK cells. He was also one of the first to describe the lytic properties of purified perforin and to postulate the synergy of perforin and granzymes, which we now know to underpin target cell apoptosis. Jurg was a major protagonist in the debate that raged throughout the 1980's and early 1990's on the physiological relevance of the 'granule exocytosis' pathway. Ultimately, resolving this issue led Jurg and his colleagues to even greater and impactful discoveries in the broader field of apoptosis research. Jurg Tschopp ranks with other pioneers, particularly Gideon Berke, Chris Bleackley, Pierre Golstein, Pierre Henkart and Eckhard Podack for making seminal discoveries on our understanding of how the immune system eliminates dangerous cells.
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Facts

- Specialised 'secretory lysosomes' store and release various potent toxins that induce the apoptotic death of dangerous cells, including those infected with a virus or undergoing malignant transformation.
- Central to the granule exocytosis mechanism is the obligate synergy between the pore forming toxin perforin and a family of serine proteases typified by the strongly pro-apoptotic granzyme B.
- Perforin is necessary for the delivery of granzyme B to the target cell cytosol where caspase-dependant and -independent pathways to apoptosis are activated.
- Jurg Tschopp and his colleagues made several seminal discoveries on the biochemical characterisation and cellular functions of perforin and the granzymes, in particular the most comprehensive and skilful characterisation of granzyme biochemistry ever undertaken.

Open Questions

- Apart from granzymes A and B, the physiological functions of many of the other granzymes remain unclear. These appear to include the production or release of pro-inflammatory cytokines from antigen-presenting cells and functions in the extracellular milieu that are independent of perforin.
- The precise mechanism by which granzymes are delivered to the target cell cytosol through perforin pores remains in dispute, with two major hypotheses still prevalent; granzymes may simply diffuse into the target cell cytosol through complement like pores or 'leak' into the cytosol following endocytic uptake and perforin-mediated disruption of endosomol trafficking.
- Pharmacological approaches that aim to enhance or inhibit perforin function may ultimately prove useful in modulating CTL/NK cell function to either promote or block killer cell cytotoxic function for therapeutic purposes.

Jurg Tschopp was responsible for numerous ground-breaking advances in the biomedical sciences, one of the first of which was when, as a young investigator, Jurg produced the definitive biochemical characterisation of the cytotoxic granules (secretory lysosomes) of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. As with many who pioneered the study of CTL/NK-induced cell death, Jurg had previously made very significant contributions to our understanding of cell lysis by complement, with the antigenic similarities between the individual membrane attack complex (MAC) components and perforin providing a vital link (see below).

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Abbreviations: CTL, cytotoxic T lymphocyte; NK, natural killer cell; MAC, membrane attack complex; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BLT, alpha-Cbz-Lys-thiobenzylester
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As with all of his other endeavours, Jurg's work showed remarkable ingenuity, and technical and intellectual rigour. Among his many strengths, Jurg Tschopp had a propensity for defining large new areas of science and coining catchy but appropriate terminology that has endured over decades. Among these, the term 'granzyme' itself was first proposed by Jurg and his colleagues.[1] Although always driven by a competitive zeal and a willingness to achieve, Jurg managed his achievements with a high degree of intellectual and personal integrity. Whenever discussing his very considerable accomplishments, I found him very honest, but at the same time modest to a fault, always placing his work into the broader context and acknowledging the good work of others.

Having commenced my own work on the mechanisms of cell death mediated by cytotoxic granules during the mid-1980s, even a cursory scan of the literature made me aware of Jurg Tschopp's capacity to massively influence the field. Despite never collaborating with Jurg Tschopp, we corresponded and met at conferences from time to time and I greatly enjoyed his hospitality in Lausanne on a couple of occasions. Jurg visited Australia quite frequently, as he has family here as well as close collaborations with colleagues at The Walter and Eliza Hall Institute. His visits provided further opportunities to meet and discuss science. On a personal level, Jurg's impact and interest on my own work can best be illustrated by relating three short vignettes. The first goes back to the mid-1980s when, as a post-doc shown into a disused laboratory at the Memorial Sloan Kettering Cancer Centre in New York with the brief to work on cytotoxic granules, I sought to gain some sort of foot-hold in the field. Papers were coming thick and fast from the laboratories of Eckhard Podack, Chris Bleackley, Irving Weissman, Pierre Henkart and John Ding-E Young, to name but a few. However, Jurg Tschopp (especially with colleagues Dieter Jenne or Daniéle Masson) simultaneously inspired and intimidated me with the scope and power of their biochemical analysis of mouse CTL granules. I recall being transfixed by a paper published in *Cell*[2] that ultimately proved to be the definitive exposé on the various members of the granzyme family. Although others were making progress on individual proteases, it was Jurg and his group who first proposed that there were enough members to constitute a family and to go about cataloguing, naming and characterising them. The meticulous 2D SDS-PAGE analyses, accompanied by definitions of proteolytic specificity and subcellular localisation, provided a young and new post-doc to New York with the scope of 'the playing field' we all shared and, although rather daunted, the beauty and rigour of the work encouraged me to persevere. This and other early work by Tschopp's group was later encapsulated in a landmark review.[3]

As detailed below, Jurg's impact on the field of CTL/NK cytotoxic granule biochemistry and function span approximately one decade, and he published around 40 papers on the subject, many of them in journals of the highest tier (Table 1). Towards the end of this period (mid-1990s), I was surprised to receive a phone call from Jurg. By this time I had returned to Australia to open my own lab at the Austin Research Institute/University of Melbourne, where I was intent on studying perforin/granzyme synergy and the means by which they co-operated to bring about target cell death. A number of landmark papers had recently emanated from the laboratories of Arnold Greenberg and Pierre Henkart that showed that applying minute concentrations of just two elements, perforin and one of the granzymes (particularly granzyme B)[4,5] or expressing them together within rat basophilic leukaemia cells[6–8] was sufficient to bring about target cell death through a non-lytic mechanism shown subsequently to be identical with 'generic' apoptosis. Jurg's question to me was simply whether my group had been able to independently reproduce these findings. I indicated that we had been able to show rapid cell death resulting from perforin combined with granzyme B, but not granzyme A. I never subsequently asked him, but I always wondered whether this conversation had any bearing on his decision to move definitively into other, more major fields of endeavour in apoptosis research and subsequently to study the molecular basis of cytokine processing and inflammation. Jurg had a 'bigger fish to fry' and while his change of scientific emphasis caused a significant void in granzyme/perforin research, a broader research community became the beneficiary of his keen scientific acumen.

Jurg's fascination with CTL/NK killing and particularly the thorny question of how perforin contributes to this process at the biochemical and cellular level continued to tantalise his curiosity for the remainder of his days. My most recent personal encounter with Jurg was at a Keystone conference in 2009, at which I was fortunate enough to present my group's recent findings on the molecular basis for perforin oligomer formation and assembly of the transmembrane pore.[9] Later during the meeting I was able to inform him that in collaboration with colleagues at Monash University and

Table 1 Milestones in discovering and characterising the granule exocytosis pathway

Year	Finding	Laboratory	Ref
1975	Granule exocytosis model proposed	Berke	11
1978	Likely involvement of proteases in CTL-mediated death and blocking by macro-molecular inhibitors	Hatcher Hudig Eisen	20–22
1980	Membrane pores on the surface of the target cell	Henkart	13
1983	First isolation of perforin, demonstration of pore-forming properties	Podack, then Tschopp	14–16
1985	First cDNA encoding a granzyme	Eisen (also Golstein, Bleackley)	25
1986	Structural, functional and antigenic similarities of perforin and complement pores	Tschopp Young	34
1986	Perforin inhibited by lipid	Tschopp	32
1986	Detailed biochemical characterisation of granzymes	Tschopp	1–3
1994	Perforin-null mice	Kagi/Hengartner (also Tschopp, Clark, Okomura)	59,62–64

Birkbeck College, London, we were about to publish the crystal structure of the perforin monomer and the cryo-electron microscopy structure of the entire perforin pore.[10] Despite the passage of so many years, Jurg seemed genuinely enthraled by the data, particularly as they finally addressed the structural similarities and antigenic cross reactivity that exists between perforin and his very first love, the complement MAC.

Jurg Tschopp–the Early Years

It was first proposed by the laboratory of Gideon Berke that target cell death could be induced through the directed exocytosis of toxic mediators secreted by killer lymphocytes.[11] This 'kiss of death' was imparted following transient conjugate formation between the two cells, leaving the target cell doomed to die, while the killer could inflict multiple further rounds of cell death on sequential targets.[12] The first observation of pore-like structures embedded on the target cell was from Pierre Henkart's group, and these complement-like pores appeared to be specifically sequestered on the target cell membrane, not that of the killer.[13] Similarity to complement lesions was also noted by Eckhard Podack and his colleagues who, with Tschopp's group were also the first to purify perforin as a 67 kDa protein and show that it was capable of inflicting osmotic lysis in its own right.[14–16] Together with previous observations that specialised cytotoxic granules became rapidly polarised towards the site of conjugate formation and the detection of the liberated proteases in the culture medium,[17–19] this early work supported a hypothesis in which pre-stored toxins liberated from a killer cell were directed specifically towards the target, at whose surface was thus delivered an irresistible death stimulus[17] (Figure 1).

In a parallel series of experiments, evidence was accumulating that proteases elaborated by the killer cell were also important in eliciting cell death. Hatcher *et al.*[20] were the first to discover that T cell-derived protease was involved in target cell death and that this effect was blocked by the serine protease inhibitor, di-isopropylfluorphosphate. The groups of Herman Eisen and Dorothy Hudig also showed that macromolecular inhibitors such as α1-antitrypsin and α1-antichymotrypsin were inhibitory when added to NK cytotoxicity assays.[21–24] Perforin has no proteolytic activity, however, cloned CTL were shown to contain and release high levels of alpha-Cbz-Lys-thiobenzylester-esterase activity,[25] subsequently shown to be because of granzymes A and K. So was born the long-standing conundrum of how these two components (a pore forming toxin and a group of proteases) could bring about the variety of cell death phenomena imparted by an intact killer cell. Despite much progress, aspects of this conundrum remain unsolved to the present day.

Jurg Tschopp's expertise in protease biochemistry and his interest in complement function (particularly the MAC) meant he was well placed to tackle the functions of perforin and the granzymes, both individually and collectively. Of course, the complement MAC works physiologically as a heteropolymeric assembly consisting of a ring of C9 molecules built onto a preformed membrane-associated complex consisting of C5b, C6, C7 and C8a-γ; in contrast, perforin forms pores on its own. Early in his career, and in conjunction with Eckhard Podack, Tschopp showed that purified C9 alone was sufficient to form transmembrane pores virtually identical to intact MAC, under certain specialised (non-physiological) conditions.[26,27] Poly (C9) was shown under electron microscopy to form tubular structures with an internal diameter of 110 Å and length of 160 Å, dimensions broadly compatible with the perforin pore.[10] Polymerisation of C9 was dependant on zinc ions, and comparison with the dimensions of the C9 monomer (also under electron microscopy) clearly indicated that a marked conformational change was required for C9 to adopt its new configuration.

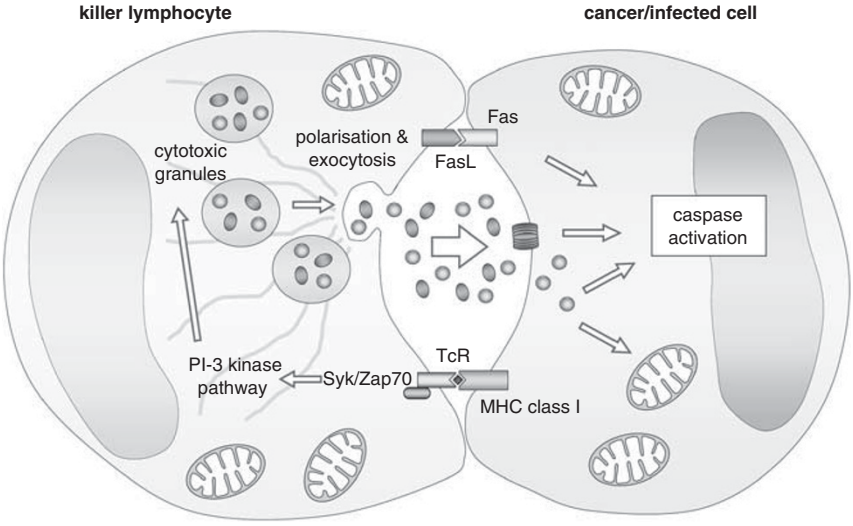

Figure 1 The granule exocytosis pathway of cell death. According to this proposed mechanism, the capacity of a killer lymphocyte (CTL or NK cell) to kill its target resides in pre-packaged toxins present in the cytoplasmic secretory granules of an activated killer cell. Upon conjugate formation, the granules became polarised close to the site of the immunological synapse, having migrated along the cell's microtubular apparatus. Upon membrane fusion and exocytosis, perforin and granzymes liberated into the synaptic cleft synergise to bring about target cell death through apoptosis. Target cell membrane disruption by perforin is an absolute requirement for access by pro-apoptotic granzymes to key substrates in a target cell cytosol, leading directly or indirectly to caspase activation

While Tschopp's group was one of the first to purify perforin and demonstrate its calcium dependant pore-forming activities, Jurg's other major early contribution was undoubtedly in unravelling and delineating the granzyme family members. Masson and Tschopp started, as did several other groups, with cytoplasmic granules isolated from many millions of killer cells on a Percoll density gradient. The removal of perforin by gel filtration then left a mixture of proteins greatly enriched for serine proteases that Tschopp proposed be designated the term 'granzymes', short for granule-associated enzymes. Cation exchange chromatography and elution with NaCl was then sufficient to focus the granzymes into six distinct clusters of protein spots on SDS-PAGE.[1,2] Apart from granzyme A, which was shown to be a disulphide-linked homodimer, each of the other granzymes collapsed down to a molecular weight of approximately 27 kDa when *N*-linked carbohydrate was removed.[3] Both granzymes A and D showed trypsin-like activity although granzyme A's activity was far stronger in cleaving the small basic synthetic substrate alpha-Cbz-Lys-thiobenzylester.[3] The pH optimum of the granzymes was around neutral, indicating that their physiological role was more likely carried out in the extra-cellular space or the target cell cytoplasm than in the acidic killer cell granules. Although Jurg and colleagues did not identify every mouse granzyme (there are up to 11 in rodents), some such as granzyme K are not abundant and others such as granzyme M are expressed only in NK cells (Table 2).

Together with other investigators, aprotinin, leupeptin and benzamidine were shown to be potently inhibitory of granzyme A, whereas antithrombin III, which is found in high concentration in plasma, was found to form irreversible inhibitory complexes with the granzyme.[28] Jurg and his colleagues also used plasmid-based expression cloning (lifts of bacterial colonies probed with rabbit anti-granzyme antisera) to isolate granzyme cDNA clones, and along with other investigators showed a close correspondence between the encoded sequences and those gleaned from more conventional protein purification and sequencing approaches. For example, the *N*-terminal sequence of mouse granzyme B agreed with the derived amino acid sequence for CCP1/CTLA-1, published from the Bleackley and Golstein labs, respectively.[3] The Tschopp lab was also the first to fish out full length cDNA clones for granzymes C, D, E and F and to show their amino acid similarity to the mast cell proteases.[29,30] A subsequent review published by Dieter Jenne and Jurg Tschopp[3] summarised these many findings, leading to a beautiful 2D-SDS-PAGE depiction of the granzyme family (reproduced here as Figure 2). In this review, Jenne and Tschopp made the prescient observation that most of the granzymes had little or no activity against synthetic oligopeptide substrates that are typically used to characterise broadly active digestive peptidases. As indicated above, granzyme A is somewhat of an exception to this 'rule'. This led them to predict 'highly specific' functions for each granzyme – in effect a processing/signalling role as distinct from broad-spectrum proteolysis. This prediction proved absolutely correct and foreshadowed (as just one example) the cell death signalling pathway so exquisitely regulated by granzyme B through its cleavage of Bid or procaspases. Tschopp later published chapters on the definitive and comprehensive study of granzyme biochemistry.[31]

The Mystery of Perforin's Physiological Function

Together with Daniéle Masson, Tschopp showed that perforin monomers could directly get inserted into a lipid, using membrane-restricted, photoactivable probes.[16] Tschopp's early observation that plasma lipoproteins[32] and proteoglycans[33] potently inactivate perforin also pointed to a mechanism

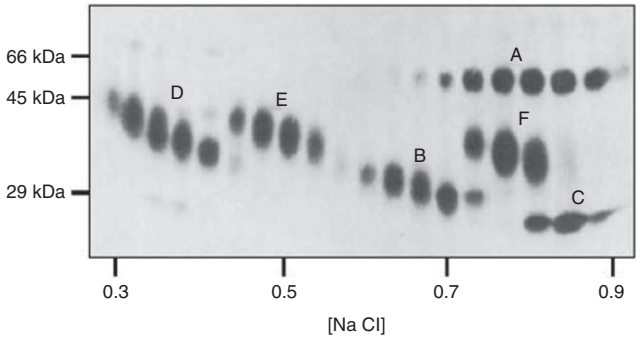

Figure 2 A typical beautiful 2-D SDS-PAGE study reproduced from an early Tschopp review showing the differential migration of the murine granzymes.[3] Superb biochemical analyses such as this proved an inspiration to the author. (reprinted by kind permission of Copyright Clearance Center Rightslink Service)

Table 2 Physical/chemical properties of granzymes in humans and rodents

Granzyme	Species	Activity	Predicted cleavage	M_r($\times 10^3$)	Cellular expression
A	Mouse, human, rat	Tryptase	Lysine, arginine	65[a]	CTLs, NK cells, $\gamma\delta$ T cells and thymocytes
B	Mouse, human, rat	Asp-ase	Aspartic acid > glutamic acid	32	CTLs, NK cells, $\gamma\delta$ T cells and thymocytes
C	Mouse, rat	Unknown	Asparagine or serine	27	CTLs
D	Mouse	Unknown	Hydrophobic	35–50[b]	CTLs
E	Mouse	Unknown	Hydrophobic	35–45[b]	CTLs
F	Mouse, rat	Unknown	Hydrophobic	35–50[b]	CTLs
G	Mouse	Unknown	Hydrophobic	30	CTLs
H	Human	Chymase	Phenylalanine > tyrosine	32	CTLs and NK cells
J	Mouse, rat	Unknown	Unknown	30	Unknown
M	Mouse, human, rat	Met-ase	Methionine > leucine	30	NK cells
N	Mouse	Unkown		30	Testis

[a]Granzyme A is the only granzyme that forms homodimers.
[b]Extensively glycosylated, particularly granzyme D.

for protecting innocent bystander cells from its marked toxicity. In a further landmark paper, Tschopp and his colleagues demonstrated the very strong overall similarity in shape and size for the cylindrical pores formed by complement and perforin, whereas there were major differences in the requirement for divalent cations and for a receptor on the target cell membrane; in particular, perforin pores did not require other further protein 'partners' and it inserted in the absence of a specific receptor on a target cell.[34] In another major contribution, the generic nature of perforin binding and membrane insertion was later shown to reside upon the simple presence of phosphorylcholine head groups on lipid, which was sufficient to enable calcium-dependant cell membrane binding to target.[35] Tschopp and his colleagues went on to demonstrate that homologous restriction factor (later known as CD59) inhibited the lytic activity of complement MAC, but not perforin.[36] It took a further 20 years for the molecular basis of this specific interaction to be understood, with CD59 shown to bind directly with residues in the membrane-spanning helices of C8 alpha.[37,38] A further mystery of perforin function (still not resolved) is why CTL/NK cells are not harmed by the perforin they secrete into the immunological synapse. Jurg's group found evidence of a specific protein within the CTL membrane with features that enabled it to block perforin function,[39] however, this was one Tschopp finding that has not found support in subsequent studies.

Investigations on the Function of Granzymes. Far from remaining restricted to pure biochemistry or cell biology, Jurg Tschopp also made significant contributions to our understanding of the regulation of expression and the function of granzymes, both in the context of target cell death and also in exploring their non-death-related (mainly extracellular) attributes. Jurg was involved in a number of collaborative studies showing the induction of granzyme gene expression and protease activity following on from lymphocyte activation with interleukin-2. It was shown that the stimulated T cells rapidly increased granzyme expression to a peak on days 3 and 4 followed by a significant decline, whereas T-cell proliferation continued exponentially for several more days.[40] With few exceptions, expression of the granzyme genes was seen only in activated T cells (particularly the CD8+ T cells) and their precursor cells in the thymus.[41] These studies also confirmed the intriguing observation that peritoneal exudate lymphocytes expressed granzymes A and B (but not perforin), while exhibiting potent cytotoxic activity (discussed further below). Later, Jurg and his colleagues produced an anti-human granzyme B antiserum that was able to detect CTL infiltrations in formalin-fixed paraffin sections of tissues from patients with cardiac allograft rejection; the first time such a marker had been used to define allo-reactive human T cells.[42] Along similar lines, Jurg was also involved in collaborative studies demonstrating the ability of granzyme A to cleave and activate the thrombin receptor expressed on neural cells, resulting in neurite retraction.[43] This finding, together with the observation that T cells found in the synovial fluid of inflamed rheumatoid joints expressed high levels of granzyme A pointed to a possible role for the granzyme in certain auto-immune diseases.[44] Of course, Jurg was to fruitfully revisit arthritis research many years later!

The Debate on Perforin's Role in Target Cell Death

Although it is now well accepted that perforin is a critical and indispensible mediator of granule-mediated cell death, the CTL/NK cell field was mired in sometimes heated debate on this issue for many years.[45–48] Despite the very strong circumstantial evidence supporting a role for granule-related mechanisms in the death of target cells, the hypothesis was vigorously challenged on a number of fronts. As already discussed, it had been known for some years that peritoneal exudate lymphocyte expressed minimal quantities of perforin, but were capable of intense cytotoxicity even in the absence of calcium that is, when EDTA was added to the culture medium. These cells and even 'conventional' CTL were also potent killers, but appeared to lack granules or the need for exocytosis when target cells were killed.[49–53] These observations were interpreted by some to indicate that perforin had nothing to do with target cell death. As ultimately proved to be the case, the data were actually pointing to a second, perforin-independent receptor-mediated pathway of cell death that could be mobilised by certain types of killer T cells. To add to the controversy, several groups showed that cells directly attacked by intact CTL or NK cells usually died by 'internal disintegration' (apoptosis) rather than osmotic lysis;[54] purified perforin was clearly a potently lytic protein that formed complement-like pores, but it was unable to induce apoptosis on its own.[55] Jurg Tschopp and his group were able to demonstrate that intact alloreactive CTL killed their targets by apoptosis, whereas the granules purified from the same cells were also capable of producing lysis under certain conditions.[56] In hindsight, this experiment had demonstrated that when an 'excessive' quantity of perforin was delivered, the lytic phenotype can become dominant over apoptosis. In fact, target cell lysis is uncommonly the consequence of attack by an intact CTL.[57]

In the final analysis, the central role of perforin in target cell death induction was universally accepted only with the demonstration that perforin-null (gene-targeted) mice are markedly deficient in inducing target cell death, severely immunosuppressed, totally lacking in NK cell cytotoxicity and susceptible to various viral infections, such as ectromelia[58] and lymphocytic choriomeningitis virus.[59] However, it took a great deal of elegant work emanating principally from the laboratories of Henkart and Greenberg to show that the principal role of perforin was to deliver the granzymes (particularly granzyme B) in such a way as to induce target cell apoptosis, rather than lysis in its own right. Central to this hypothesis was the observation that granzyme B is the only mammalian serine protease capable of cleaving substrate proteins specifically after highly specific aspartate residues,[60] a property that later defined the cysteine protease caspases. As discussed above, the application of purified granzyme B (alone) to target cells proved innocuous, whereas its combination with very small quantities of perforin resulted, not in lysis, but in classic apoptosis. This and experiments by Henkart with rat basophilic leukaemia cells induced to express perforin and/or granzymes A/B pointed to specific

pro-apoptotic synergy between the two distinct types of toxin. Although perforin gene-deleted mice were first reported in 1994, Jurg Tschopp utilised antisense oligonucleotides to induce partial perforin 'knock-down' (and to effectively predict the outcome of perforin gene deletion) some years earlier.[61] In effect, Jurg and his team demonstrated that reduced perforin expression resulted in an approximately proportional reduction in target cell death, providing the first direct evidence for perforin's critical role in the process.

In the end, Jurg Tschopp and his collaborators were not the first to report perforin-deficient mice, this distinction belonging to David Kagi and Hans Hengartner.[59] It was a desperately close race! During 1994, three further groups headed by Jurg Tschopp, Bill Clarke and Ko Okomura all reported the profound immunosuppression and inability to clear virus-infected cells associated with perforin deficiency.[62–64] At about the same time, the cloning of Fas/CD95 and its membership of the tumour necrosis factor superfamily provided an explanation for the residual, but still significant target cell death that remained in perforin-deficient mice. Experiments from the Golstein laboratory showed that engagement of CD95 with its ligand expressed on activated T cells provided the second major pathway to target cell apoptosis.[65,66] Delineation of the FasL/Fas pathway explained the target cell death induced by peritoneal exudate lymphocyte, as this mechanism remains active in the absence of calcium, as had been observed by Berke and other scientists.

Conclusion

As with his other fields of interest, Jurg Tschopp made many enduring and invaluable contributions to the study of CTL/NK-induced target cell death. His insights, energy and enthusiasm for science, and for life itself will be sorely missed by all.

Conflict of Interest

The author declares no conflict of interest.

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