

An early history of T cell-mediated cytotoxicity

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Abstract | Sixty years of intense fundamental research into T cell-mediated cytotoxicity have led to detailed knowledge of the cells involved, of specific recognition mechanisms, and of post-recognition perforin–granzyme-based and FAS-based molecular mechanisms. What could not be anticipated at the outset was how discovery of the mechanisms regulating the activation and function of cytotoxic T cells would lead to new developments in cancer immunotherapy. Given the profound recent interest in therapeutic manipulation of cytotoxic T cell responses, it is an opportune time to look back on the early history of the field. This Timeline describes how the early findings occurred, eventually leading to current therapeutic applications.

Initially, scientists investigated cell-mediated cytotoxicity in their efforts to understand graft rejection. They also wondered why a cytotoxicity mechanism should have been evolutionarily selected in multicellular organisms, in which cytotoxic cells could kill only other cells of the same organism. This seemed to negate the fact that multicellularity depends on cooperation between cells. We now understand that cytotoxicity defends against those **syngeneic [G]** cells — either modified by oncogenesis or bearing intracellular infectious agents that are not otherwise reachable — whose death would benefit the whole multicellular organism. The same mechanisms are also thought to be involved in artificial conditions such as allograft rejection or in pathological circumstances such as in some autoimmune diseases.

This Timeline article covers sixty years of fundamental research into T cell-mediated cytotoxicity, starting with early in vitro demonstrations of cell-mediated cytotoxicity, the quantification thereof and its attribution to T cells. This was followed by more analytical investigations, by which recognition of target cells was shown to involve specific T cell surface receptors, and post-recognition cytotoxicity was shown to be due to either a perforin- and granzyme-based mechanism or a FAS-based mechanism (**FIG. 1**). Importantly, **we**

~~believe that~~ currently emerging therapeutic applications of T cell-mediated cytotoxicity, such as cancer immunotherapy, **may** benefit from a consideration of its mechanistic bases.

Although natural killer (NK) cells and some CD4⁺ T cells are also endowed with cytotoxic activity, here we mainly discuss cytotoxicity mediated by ‘classical’ cytotoxic CD8⁺ T cells (also known as cytotoxic T lymphocytes (CTLs)), as has been studied most often in vitro. We focus on what we consider retrospectively to be the major advances in the field and apologize for the omission of many other excellent studies out of the several tens of thousands that have been published on this topic.

[H1] Recognizing cytotoxicity: 1960s

Pioneering experiments in the 1950s that aimed to find a mechanistic explanation for graft rejection assessed, mostly by microscopy, the cytolysis of donor tissue by sensitized lymphoid tissue in vitro. However, these experiments showed no evidence of cytotoxicity, perhaps because they were often carried out on donor explants rather than on isolated cells and also because they often aimed to show the participation of circulating antibodies and/or complement in these reactions (reviewed in REFS¹⁻³).

[H2] Early demonstrations of cytotoxicity.

The first reports, in the 1960s, to show specific cytotoxicity in vitro used mostly **allogeneic [G]** combinations of cells. Thus, lymphocytes from dogs bearing a kidney **homograft [G]** produced specific lesions in donor kidney cultures, as visualized microscopically¹. Similarly, lymphocytes from BALB/c mice alloimmunized with cells from C3H mice were shown to cluster on target cells of C3H origin and to induce their cytolysis. These target cells withdrew their processes and rounded up, with evidence of chromatin clumping that is suggestive, in retrospect, of apoptotic cell death². Also, lymph node or thoracic duct lymphoid cells from allosensitized rodents were able to cluster around and destroy donor kidney cells in culture⁴.

Cytotoxicity mediated by alloimmune mouse lymphoid cells could also be demonstrated by **clonogenic assays [G]**⁵ and through the inhibition of **antibody plaque formation [G]**⁶. Mouse alloimmune peritoneal cells — collected 10 days after an intraperitoneal injection of allogeneic tumour cells — comprising 3–5% lymphocytes showed strong immediate allospecific adherence and cytotoxicity **to** fibroblast monolayers, as demonstrated by the formation of clear plaques⁷. Cytotoxicity mediated by mouse alloimmune lymph node cells could be partially blocked by alloantibodies⁸.

In a **xenogenic [G]** combination, lymphoid cells from mice immunized with human HeLa cells were cytotoxic to monolayers of HeLa cells⁹. Probably distinct from the above mechanisms, some target cells, such as L cells or embryonic fibroblasts, were shown to be sensitive to toxic substances (later defined as tumour necrosis factor or lymphotoxin) released by activated lymphocytes in an antigen-independent manner^{10,11}.

Other experiments were designed to mimic syngeneic autoimmune reactions, initially using interspecies immunization. Thus, lymph node cells from Lewis rats immunized with a suspension of guinea pig spinal cord were added to dog brain cells in vitro. These sensitized rat cells (but not non-sensitized cells) agglutinated around dog glial cells (but not dog fibroblasts) and destroyed them, thus demonstrating tissue-specific cytolysis in vitro¹². Somewhat similarly, white blood cells or thoracic duct lymphocytes from rabbits or rats immunized with pig spinal cord agglutinated around and ultimately destroyed rat glial cells¹³.

[H2] Quantifying cytotoxicity.

By the mid-1960s, it was clear, at least qualitatively, that **sensitized** lymphoid cells could lyse target cells in vitro. Further studies on the mechanisms of this cell-mediated cytotoxicity required better quantification. At about this time, several publications noted that the release from target cells of pre-incorporated radioisotopes, such as ³²P orthophosphate or ¹⁴C amino acids, could be used as an indicator of cell damage^{14,15}.

Most subsequent cytotoxicity experiments were carried out using the **51Cr release assay [G]**. For nucleated target cells, this method was first used to evaluate the effect of antibodies in the presence of complement¹⁶⁻¹⁸ and was subsequently applied to cell-mediated cytotoxicity^{19,20}. This method for assessing cytotoxicity had the advantages of being objective, short-term, accurate (early users were surprised by the low dispersal of experimental triplicates) and, because of the ⁵¹Cr half-life of 27 days and convenient gamma emission, more user-friendly than the use of other radioisotopes such as ³H or ³²P.

The ⁵¹Cr release assay was extensively used until the mid-1990s and is now being progressively replaced, in particular for safety reasons, by non-radioactive methods. These include the release from dying target cells of lactate dehydrogenase (LDH), calcein or cytokeratin, the uptake by dying target cells of propidium iodide or similar normally cell-impermeant dyes, the labeling in or on dying cells of activated caspases or exposed phosphatidylserine²¹⁻²³ and more sophisticated microscopy methods (see below).

[H1] Identifying CTLs: 1970s

By the end of the 1960s, the accumulation of functional results on lymphoid cell-mediated cytotoxicity (reviewed in REF.²⁴) and the emergence of appropriate methods to quantify such cytotoxicity enabled further analysis of the cells involved and their recognition mechanisms.

[H2] T cells are required for anti-allogeneic cytotoxicity.

Researchers were keen to discover which cells **in-particular** are able to exert cytotoxicity in alloimmune spleen or lymph node cell populations. This was first investigated by treating such populations with T cell-specific antisera and then testing their residual cytotoxicity. In the presence of complement, but usually not in its absence, antisera such as anti-Thy1 (REFS²⁵⁻²⁷), heterologous anti-T²⁸ and most notably anti-Ly23 (REF.²⁹) (Ly23 being later known as CD8) largely abolished subsequent anti-allogeneic cytotoxicity of mouse cells. This showed that such cytotoxicity requires T cells.

[H2] T cells are sufficient for anti-allogeneic cytotoxicity.

The demonstration that T cells are not only required but also sufficient for cytotoxicity was provided by using T cell-rich populations, such as thymus cells, that were allosensitized either in vivo or in vitro and then subjected to various methods for the depletion of non-T cells before testing for cytotoxicity^{25,27,28,30-33}. A main conclusion from these studies was that a very small number of highly purified T cells were still able to exert cytotoxicity²⁷, which makes it unlikely that non-T cells take part in the effector phase of **T cell-mediated** cytotoxicity. This autonomy of CTLs at the effector stage was consistent with the visualization of cell conjugates that often contain only one effector cell killing one target cell (see below).

[H2] Early attempts to demonstrate specific surface receptors.

Thus, by the early 1970s, it was clear that some T cells can autonomously induce target cell death. Analytical methods were then required to begin to dissect the mechanism(s) of this cytotoxicity. A good starting point was the hypothesis, discussed by D. Bernard Amos in the conclusion of an extensive review in 1962 (REF.³), that the specificity of cytotoxicity is the result of a specific recognition step (for example, through Paul Ehrlich's so-called 'sessile antibodies' on the cell surface) followed by non-specific cytotoxicity, rather than of specificity of the cytotoxic mechanism per se. Thus, specific recognition of target cells by CTLs putatively implied the existence of specific T cell surface receptors, which was also relevant to the then ongoing search for the T cell receptor for antigen³⁴. Of note, columns of

antigen-coated beads retained antigen-specific B cells, thus demonstrating the existence of specific receptors for antigen at the B cell surface³⁵, but could not retain antigen-specific T cells³⁶. It was then realized that the recognition of antigen by T cells requires the presentation of this antigen through MHC molecules at the cell surface³⁷⁻⁴⁰; this was an important finding by itself and one of the key discoveries in T cell biology, **and** it also explained why antigen-coated beads failed to specifically retain antigen-specific CTLs, whereas cell monolayer techniques (detailed below) succeeded.

[H2] Specific adsorption of CTLs on cell monolayers.

Early experiments, mentioned above, had already shown that **sensitized** lymphocyte populations contain cells that specifically aggregate on relevant cell monolayers. However, these results did not show that the aggregating cells were the same as the cytotoxic cells. A pioneering systematic investigation of this issue was carried out by Boris Brondz and colleagues, working in then relative isolation at the Gamaleya Institute in Moscow, initially in Lev A. Zilber's laboratory. Lymphocytes from in vivo alloimmunized mice, showing specific cytotoxicity in vitro against target cells bearing the immunizing H2 MHC antigens⁴¹, were incubated on macrophage monolayers and the non-adherent cells were then tested for specific cytotoxic activity. Cytotoxic activity of non-adherent cells was lost if incubation had been on monolayers bearing the immunizing H2 alloantigens, which strongly suggested that the cytotoxic cells were adsorbed on these monolayers because they bore surface receptors recognizing these antigens⁴². These studies also included investigations of the precise specificity of H2 **congenic [G]** alloreactions and their implications regarding the existence, nature and specificity of T cell receptors^{42,43} (reviewed in REF.⁴⁴). One cannot be but impressed by Brondz' published work in terms of the quality of his experiments and discussion, **in particular** in the light of the few contacts that were available with Western scientists at that time.

The specific loss of cytotoxic activity by adsorption of alloimmune mouse lymphocytes on relevant cell monolayers could be reproduced in a more convenient and quantitative manner soon afterwards using fibroblast monolayers and a 51Cr release assay^{45,46}, also making use of highly purified T cells²⁸. Moreover, after the incubation of alloimmune lymphocytes on relevant fibroblast monolayers, not only did non-adsorbed lymphocytes lose cytotoxicity, but also adsorbed lymphocytes could be recovered from the adsorbing monolayers and shown to be cytotoxic. This showed that the loss of cytotoxic activity of adherent cells was due to specific cell adsorption rather than specific inactivation^{45,46}. The

existence of specific receptors for alloantigens was similarly demonstrated on normal mouse lymphocytes prior to sensitization⁴⁷. Together, these experiments showed the existence of specific receptors for antigen at the surface of CTLs and their precursors.

[H2] Specific CTL–target cell conjugates.

Conjugates formed between one effector cell and one target cell could be obtained⁴⁸ using tumour target cells and cytotoxic alloimmune lymphocytes from peritoneal exudate⁴⁹. Study of these conjugates confirmed that a single CTL was required and sufficient to kill a target cell and that a CTL could recycle, which showed that effector cells do not die when killing target cells⁴⁸. By microcinematography, **sensitized** lymphocytes were seen to approach the target cell, form a conjugate and then leave the target cell. The target cell burst after a delay, whereas the cell that had killed it was able to approach and kill other target cells^{50,51}. Electron microscopy also showed the close apposition of effector and target cell membranes during conjugate formation⁵¹.

[H2] Linking killing and dying.

A striking observation made by Colin Sanderson using microcinematography was that, after being exposed to CTLs, dying target cells showed cell surface blebbing, which was distinct from the events that occur during complement-mediated lysis⁵² and similar to the apoptotic cell death that had recently been described in detail⁵³. Importantly, this suggested that CTLs did not kill the target cells directly, but signalled to them to undergo cell death using their own self-destruction machinery⁵². Also, if — as discussed above and in line with earlier results — the specific recognition of target cells and cell death signalling were separate steps, then these studies suggested that the post-recognition death signals need not be antigen specific. However, the death signals would need to somehow be polarized towards the target cells, as CTLs were not killed when killing target cells despite being sensitive to killing by other CTLs⁵⁴.

These and further more detailed phenomenological advances obtained through diverse analytical methods^{55,56} were abundantly reviewed at the time⁵⁷⁻⁶⁵. An important development during this period was the ability to clone CTLs⁶⁶⁻⁶⁸. These advances set the stage in the early 1980s for researchers to begin to determine the post-recognition mechanism(s) that are involved in target cell killing (**FIG. 2**).

[H1] Cytotoxic mechanisms: 1980s–1990s

Identification of post-recognition mechanisms was made possible in particular by the emergence at that time, in this field, of molecular biology approaches such as transfection, subtractive cDNA cloning and gene inactivation.

[H2] The perforin–granzyme pathway.

Microscopic observations provided a first hint as to the possible nature of a post-recognition mechanism of cytotoxicity. Tubular lesions were observed in target cell membranes after incubation with cytotoxic cells^{69,70} and also with purified granules from various types of cytotoxic **cells**⁷¹⁻⁷³. A cytolytic protein known as perforin could be isolated from these granules⁷⁴⁻⁷⁷ and was found to be homologous to the ninth component of complement⁷⁸. These important findings were in line with a granule-exocytosis model of cytotoxicity proposed by Pierre Henkart, who hypothesized that "target cell binding to a membrane receptor induces a secretory process in the effector cell in which the contents of cytoplasmic granules are released by local exocytosis between the effector cell and its bound target"⁷⁵. Such a model implied that effector cell granules, containing perforin **eventually** together with other required molecules, were delivered to the target cell. The involvement of perforin in a major mechanism of cytotoxicity was confirmed a few years later by studies showing the marked decrease in cytotoxicity mediated by CTLs transfected with perforin antisense material⁷⁹ or CTLs from perforin-knockout mice^{80,81}.

However, CTLs had been shown previously to induce apoptosis, whereas purified perforin induced a different type of cell death similar to necrosis. This suggested at the time that perforin could not be the only effector molecule of target cell death and that it had to function in association with other molecules to induce apoptosis. Serine-esterases were likely candidates, as serine-esterase inhibitors inhibited T cell-mediated cytotoxicity^{82,83}. Indeed serine-esterases could be biochemically isolated from CTLs⁸⁴⁻⁸⁷ and moreover were localized in their granules⁸⁸⁻⁹⁰, hence being named 'granzymes'.

Granzymes were independently identified through a subtractive cDNA cloning approach, based on the rationale that **some of the** molecules involved in cytotoxicity may be expressed only in cytotoxic cells. **Such molecules** with a restricted tissue distribution were identified by subtracting mRNAs from non-cytotoxic cells from CTL mRNAs, and cloning the resulting material (reviewed in REF.⁹¹). This led to the identification of CTLA3 (REFS^{92,93}), which was identical to the independently identified Hanuka factor⁹⁴ and granzyme A; and of CTLA1 (REF.⁹²), which was identical to the independently identified CCP1 (REF.⁹⁵) and granzyme B.

The role of granzymes in cytotoxicity was not easy to formally establish through gene inactivation because of functional redundancy. However, such a role was strongly suspected on the basis of, **in particular**, the apoptosis-like cytotoxicity that was observed in perforin–granzyme co-transfection experiments⁹⁶⁻⁹⁸. It later became clear that, usually with the help of perforin, effector cell granzymes can activate distinct death pathways within the target cell and can have multiple roles⁹⁹⁻¹⁰³.

[H2] The perforin-granzyme pathway does not fully account for cytotoxicity.

Thus, by the late 1980s, a major granule exocytosis mechanism of CTL-mediated cytotoxicity was strongly suspected to involve both perforin and granzymes, first localized within CTL granules, then somehow exocytosed, to enter the target cell and cause its death by triggering apoptosis. However, in parallel, arguments were lining up to suggest that this mechanism did not fully account for cytotoxicity. For example, peritoneal exudate lymphocytes that seemed to lack both granules and significant levels of perforin expression were highly cytotoxic¹⁰⁴, which lead researchers to discuss alternative explanations for cytotoxicity including, in particular, cell surface death receptors¹⁰⁵. Also, granule exocytosis and perforin-based cytotoxicity required Ca^{2+} , but part of the cytotoxic activity of CTL populations or clones was found to be independent of extracellular Ca^{2+} (REFS¹⁰⁶⁻¹¹⁰).

Scientific meetings discussing cytotoxicity were particularly lively at that time, with heated controversies between proponents of perforin-only, not-only-perforin and no-perforin mechanisms. Even written comments on this debate had less-than-detached titles¹¹¹.

[H2] Identifying the FAS pathway.

If CTLs were indeed using two distinct mechanisms of cytotoxicity, then studying one of these mechanisms might benefit from excluding the other. One way to achieve this was to attempt to delete the known granule exocytosis mechanism from CTLs, for example through loss of genetic material. The PC60 T cell hybridoma initially derived by Markus Nabholz, which was constitutive for growth and inducible for cytotoxicity upon addition of interleukins¹¹², seemed a good starting point. PC60 is a heterospecific hybridoma between rat and mouse cells, and it was known that such hybridomas tend to randomly lose mouse or rat chromosomes. PC60 cells were grown in long-term culture and periodically cloned by limiting dilution (described in detail in REF.¹¹³). These clones were tested for their cytotoxicity against various target cells, with the clone showing highest levels of cytotoxicity being expanded, tested cytogenetically, grown again for several months, then cloned again,

and so on. This process was repeated for several years, leading to a tenth-generation PC60-d10S derivative. These d10S cells had lost about 20 chromosomes from the initial sum of the parental chromosomes. They showed non-MHC-restricted, Ca^{2+} -independent cytotoxicity. When d10S cells were tested against a range of target cells, thymocytes were particularly sensitive¹¹⁴.

While these results were being obtained, a striking report by Shigekazu Nagata and colleagues¹¹⁵ mentioned that FAS (also known as APO-1 or CD95), a known cell-death-transducing surface molecule^{116,117}, was abundantly expressed on thymocytes of wild-type mice but was not detectably expressed in *lpr* mice [G]¹¹⁵. These results led to speculation that FAS could be involved in d10S-mediated cytotoxicity and offered a way to test this hypothesis. Indeed, whereas wild-type mouse thymocytes were sensitive to d10S-mediated cytotoxicity, *lpr* mouse thymocytes were not. Also, FAS-negative tumour target cells became sensitive to cytotoxic killing by d10S cells upon transfection of FAS¹¹⁴. This FAS-based mechanism of cytotoxicity was not limited to PC60-d10S cells, as the Ca^{2+} -independent component^{106,107} of antigen-specific cytotoxicity mediated by alloimmune peritoneal exudate cells lysed FAS-bearing, but not FAS-deficient, target cells¹¹⁴. In the summer of 1992, the report describing these results was rejected by *Nature*, **but it was** rapidly accepted elsewhere and published in January 1993 (REF.¹¹⁴). These results showed that a mechanism of T cell-mediated cytotoxicity was FAS dependent.

[H2] Two pathways for cytotoxicity, and identification of the FAS ligand.

These results were further developed in two directions. First, in terms of cytotoxicity, it was shown that CTLs from perforin-knockout mice could still lyse target cells, through the FAS pathway, and that target cells from *lpr* mice could still be lysed by the perforin–granzyme pathway but, importantly, could not be lysed by perforin-knockout effector cells. These and other similar experiments showed that only two main mechanisms accounted for T cell-mediated cytotoxicity, namely the perforin–granzyme-dependent granule exocytosis mechanism and the FAS-mediated mechanism¹¹⁸⁻¹²¹.

Second, at that time several groups were trying to identify the ligand for FAS. It was reasoned that d10S cells, which exerted cytotoxicity through FAS expressed on target cells, must bear a ligand for FAS. The Nagata laboratory used a FAS-Fc construct to further FACS-select d10S cell derivatives overexpressing the putative FAS ligand. They succeeded to clone the FAS ligand (FASL; also known as CD95L) from these cells by expression cloning and published the corresponding report in December 1993, within one year of receiving the d10S

cells¹²². These and other early developments of FAS research were extensively reviewed at the time (for example, see REF.¹²³). Further studies showed that the perforin–granzyme pathway seems to be the dominant pathway for killing infected or transformed cells, whereas the FAS pathway seems to be more involved in regulating lymphocyte homeostasis¹²⁴.

Altogether, by the mid-1990s, the broad outlines of the FAS-based and the perforin–granzyme-based mechanisms by which CTLs can induce target cell death had been established. As a consequence, these years constituted a turning point for researchers in the field. Some turned to the study of cell death per se, whereas others went on studying, in greater molecular detail, the mechanisms of CTL-mediated cytotoxicity (**FIG. 2**).

[H1] Perforin-granzyme-cytotoxicity: 1990s

In CD8⁺ T cells, the expression of perforin, granzymes and FASL was shown to be upregulated by, in particular, the transcription factors BLIMP1, eomesodermin and T-bet, through CD4⁺ T cell help in conjunction with changes to T cell metabolism (reviewed in REFS¹²⁵⁻¹²⁸). As revealed by immuno-electron microscopy in both NK cells and CTLs, perforin and granzymes co-localize with lysosomal hydrolases in a dual-function organelle that functions as both a lysosome and a secretory granule, being known as a secretory lysosome^{129,130}. This raised the interesting question of how CTLs have evolved to turn a lysosome into a secretory organelle. A key finding emerged from genetic analysis of the primary immunodeficiency **haemophagocytic lymphohistiocytosis [G]** (HLH), which is a rapidly fatal disease in which CTLs are unable to kill. Importantly, not only mutations in perforin¹³¹, but also in other molecules¹³², led to HLH. The latter mutations were in genes encoding the proteins RAB27A, UNC13D (also known as MUNC13-4), syntaxin 11 and MUNC18-2 (also known as STXBP2)¹³², which are required for secretion of the modified CTL lysosome.

An early indication of how CTL activation leads to granule secretion was provided by the observation of an increase in cytosolic Ca²⁺ upon target cell recognition¹³³. Following early observations in CTLs and NK cells of polarization of the Golgi apparatus and the microtubule-organizing centre towards target cells¹³⁴⁻¹³⁶, confocal imaging revealed the remarkable reorganization of receptors that occurs at the interface between T cells and antigen-presenting cells¹³⁷. This led to the discovery of a specialized secretory domain within the corresponding ‘immunological synapse’ of CTLs, where cytolytic granules deliver their cargo to target cells¹³⁸. Further studies revealed a novel role for the centrosome in docking directly with the plasma membrane at the immunological synapse, thereby focusing secretion

at the point of TCR signalling¹³⁹ and ensuring that only the recognized target cells are killed. Ultimately, the granule contents — including perforin, granzymes and also the antimicrobial peptide granulysin (expressed in humans, but not rodents) — ended up in the intercellular cleft between the CTL and target cell.

At the level of the target cell, perforin was required but not sufficient for lysis. Perforin was shown to facilitate access to the cytosol of death-triggering granzymes, by forming pores at the cell membrane^{96,140}. Several granzymes could induce apoptosis, but granzyme B seemed to be the most efficient by targeting caspases either directly or through the pro-apoptotic protein BID¹⁰¹. Several granzymes could also lead to non-apoptotic forms of cell death⁹⁹. Moreover, granzymes and granulysin could target and destroy intracellular parasites¹⁴¹⁻¹⁴⁴.

[H1] FAS-based cytotoxicity: mid-1990s

One puzzle regarding FAS-based killing arose from the observation that FASL activity was rapidly inactivated by metalloprotease cleavage of the extracellular domain at the plasma membrane¹⁴⁵. Although this showed that FASL activity required membrane association and revealed a clear mechanism for down-regulation of FAS-mediated killing, it also raised the question of how FASL might be active even transiently once at the plasma membrane. One possible answer is that FASL is packaged within cytotoxic granules in CTLs and NK cells¹⁴⁶. Moreover, ubiquitylation signals in the cytosolic tail of FASL target it to intraluminal vesicles within such cytotoxic granules¹⁴⁷, which is consistent with the observation of FASL activity within vesicle preparations¹⁴⁸. Thus, FASL is found in intraluminal vesicles that surround the perforin-containing dense core within cytotoxic granules¹⁴⁹. FASL-bearing vesicles would be released from cytotoxic granules upon secretion into the small cleft formed between the CTL and the FAS-bearing target cell to provide highly focused FASL-mediated killing.

In the target cell, as a FASL-constrained functional trimer¹⁵⁰, FAS was shown to signal to FAS-associated death domain protein (FADD)¹⁵¹, leading to caspase 8 activation¹⁵² then caspase 3 activation and apoptosis. FAS-induced signalling was shown to be regulated in part by molecular recruitment to membrane rafts¹⁵³ and by post-translational modifications¹⁵⁴. FAS signalling also induced various functions in addition to cell death¹⁵⁵⁻¹⁵⁸. The lymphadenopathy that is prominent in mouse and human¹⁵⁹ mutants of the FAS pathway indicates that a main function of this pathway is to regulate the immune system, classically through the control of lymphocyte death although other mechanisms have been proposed^{158,160}.

[H1] CTLs and therapy

The knowledge that was accumulated over many years of research on T cell-mediated cytotoxicity started to lead to therapeutic applications, for example through the molecular definition of the cytotoxicity mutants described above. In parallel, other developments were taking place that also turned out to be relevant to the role of CTLs in therapy. Perforin-deficient mice were shown to be more susceptible than wild-type mice to carcinogenesis, which implicated the immune system, and more precisely CTLs, in cancer immunosurveillance^{161,162}. Cancer immunotherapy was attempted using IL-2-activated lymphocytes¹⁶³. In 1987, the immune checkpoint molecule cytotoxic T lymphocyte antigen 4 (CTLA4) was isolated through subtractive cDNA cloning^{91,164}, and about 5 years later another checkpoint molecule, programmed cell death protein 1 (PD1), was similarly identified¹⁶⁵. *Ctla4*-knockout mice^{166,167} and humans with *CTLA4* mutations^{168,169} have similar lymphoproliferative phenotypes, which showed that CTLA4 has an inhibitory effect on lymphoid cells. Similarly, although not exactly the same, *Pd1*-knockout mice had splenomegaly, abnormalities of B cell proliferation and differentiation¹⁷⁰ and lupus-like autoimmune disease¹⁷¹, which suggested that PD1 maintains peripheral self-tolerance by negatively regulating immune responses¹⁷². The different phenotypes resulting from CTLA4 or PD1 deficiency or mutation reflect the fact that they function, at least in part, at distinct steps and also on distinct T cell subsets¹⁷³. If CTLA4 and PD1 negatively regulate the activation of CTLs, contributing to their exhaustion¹⁷⁴, then it was proposed that interfering with these molecules, using for example specific antibodies, could (re)activate CTLs to help eliminate or at least control tumour growth. Indeed, in tumour-bearing mice, antibodies directed against CTLA4 (REF.¹⁷⁵) or the PD1–PDL1 pathway^{176,177} could lead to tumour regression. Striking remissions were observed in a minority of patients with melanoma who were treated with antibodies against CTLA4 (REF.¹⁷⁸) or PD1 (REFS^{179,180}). Many clinical trials using such antibodies to treat other types of cancer are underway¹⁸¹. Although the possibility has not yet been fully explored, CTL activation using antibodies against such inhibitory checkpoint molecules may also have potential to be used to fight viral, bacterial and parasitic infections^{182,183}.

The alternative possibility arising from the discovery of checkpoint molecules was the ability to decrease CTL activity in certain therapeutic or pathological circumstances. Soluble CTLA4–Fc constructs can engage CD80 and CD86 (which are ligands of both CTLA4 and CD28) with high affinity, leading to inhibition of the CD28-mediated co-stimulation pathway

and consequent inhibition of CTL activation^{184,185}. Such constructs were indeed shown to be efficient against some autoimmune diseases¹⁸⁶ and against graft rejection^{187,188}. A possible similar use of PD1 agonists is also being considered¹⁸⁹.

CTLs have also been effectively harnessed in other ways to control cancer. Instead of activating endogenous CTLs through the use of injected antibodies as described above, CTLs themselves can be injected into a patient¹⁹⁰, in particular after their transfection with genes encoding chimeric antigen receptors (CARs)^{191,192}. These CARs consist of an antibody-derived targeting fragment together with signalling domains that can activate T cells. Ideally, the antibody-derived fragment is selected to recognize, in an MHC-independent manner, an antigen that is present at the surface of cancer cells but not healthy cells. The signalling domains thus lead to activation of the CTLs, which then induce cancer cell death. Such CAR T cells have already shown striking efficacy at controlling some haematological malignancies¹⁹³.

[H1] Conclusions

Sixty years of research have led to the characterization of CTLs and mechanistic investigation of their cytotoxic effects. Perhaps the most thought-provoking result of these studies has been the realization that CTLs most often kill target cells by inducing signalling through the same apoptotic pathway that is used for developmental cell death. Within the past 30 years, further molecular investigations have led to existing or promising potential clinical applications for some genetic diseases, autoimmune and infectious diseases and graft rejection, and perhaps most importantly for cancer immunotherapy using CTLs as a therapeutic tool. Research in the field of CTLs may now have reached the point where it has progressed beyond basic science and is dominated by medical applications, hopefully leading to further exciting therapeutic advances.

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Author contributions

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Competing interests statement

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Figure 1 | **Timeline of the history of research on cytotoxic T cells (CTLs).** CAR T cell, chimeric antigen receptor-bearing T cell; CTLA4, cytotoxic T lymphocyte antigen 4; PD1, programmed cell death protein 1.

Figure 2 | **Chronological scheme of research on the mechanisms of CTL-mediated cytotoxicity.** A cytotoxic T lymphocyte (CTL) recognizes through its T cell receptor (TCR) an antigen–MHC complex on a target cell. This can trigger the migration of granules towards the region of the CTL facing the target cell. **The figure shows a simplified representation of granules, which are known** to contain both FAS ligand (FASL) on intraluminal vesicles and perforin and granzymes¹⁴⁹. Alternatively, other studies have suggested that FASL is contained in distinct granules **for** perforin and granzymes¹⁹⁴. Granules within the CTL open into the intercellular cleft (the immune synapse) upon CTL activation, where they release their contents. This can lead to two distinct mechanisms of cell death in the target cell. In one mechanism, FASL engages FAS at the target cell membrane, leading, through FAS-associated death domain protein (FADD), to the activation of caspase 8. This leads, in turn, to the cleavage and thus activation of caspase 3 and subsequent apoptosis. In the other mechanism, perforin opens a channel in the target cell membrane, through which granzyme B enters the cytosol and activates caspase 3, also leading to apoptosis. Less frequent, non-apoptotic types of cell death have also been described following encounters with CTLs. The colour scale corresponds to the date of publication of discovery or use of the corresponding moiety. Note that most of the studies on the perforin–granzyme-mediated mechanism of cell death preceded those on the FAS-mediated pathway.

Glossary

51Cr release assay

Evaluates the percentage of target cells that are lysed by cytotoxic T cells by measuring the proportion of radioactivity released from pre-labelled target cells.

Allogeneic

Describes tissues or cells that are of the same species but are not genetically identical.

Antibody plaque formation

The ability of haemolytic antibody-forming lymphocytes to form plaques of lysed red blood cells in agar after being subjected to, for example, cytotoxic T cells.

Clonogenic assays

Determine the percentage of cells that are able to form colonies in vitro after being subjected to, for example, cytotoxic T cells.

Congenic

Describes tissues or cells that genetically differ by only one chromosomal region.

Haemophagocytic lymphohistiocytosis

(HLH). A human autosomal recessive disorder that results from mutation of one of five genes, including the gene encoding perforin, and that leads to T cell hyperproliferation.

Homograft

A graft from a donor of the same species as the recipient.

***lpr* mice**

Mice bearing the *lpr* mutation of the *Fas* gene, leading to a lymphoproliferative phenotype.

Syngeneic

Describes tissues or cells that are genetically identical.

Xenogeneic

Describes tissues or cells that are of different species.

Online only**Subject categories**

Biological sciences / Immunology / Lymphocytes / T cells / CD8-positive T cells / Cytotoxic T cells

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Biological sciences / Immunology / Immunotherapy

[URI /631/250/251]

ToC blurb

This Timeline article looks back at the past sixty years of fundamental research into the mechanisms of T cell-mediated cytotoxicity, which has culminated in recent interest in the therapeutic manipulation of cytotoxic T cell responses for cancer immunotherapy.

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Fig 1

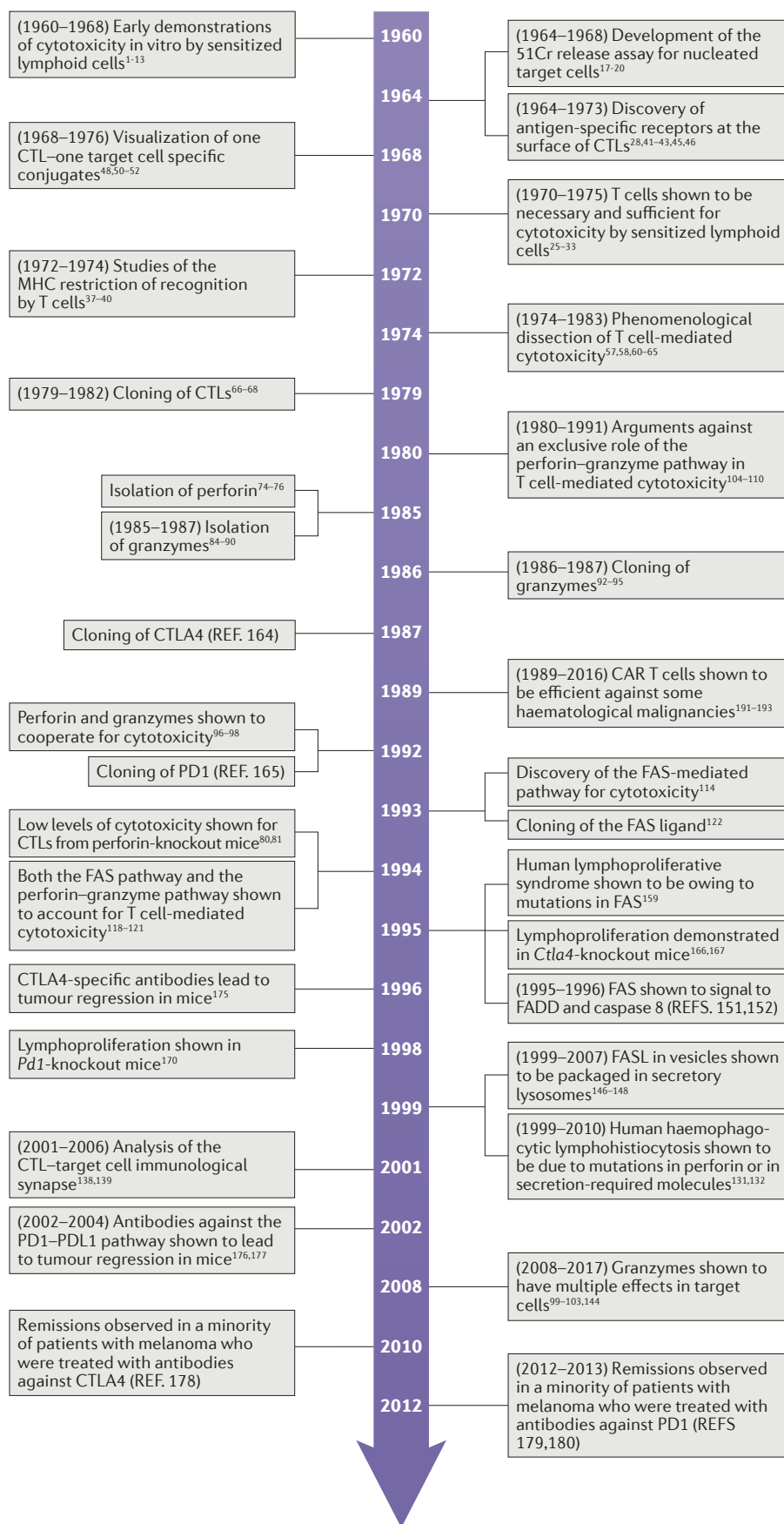
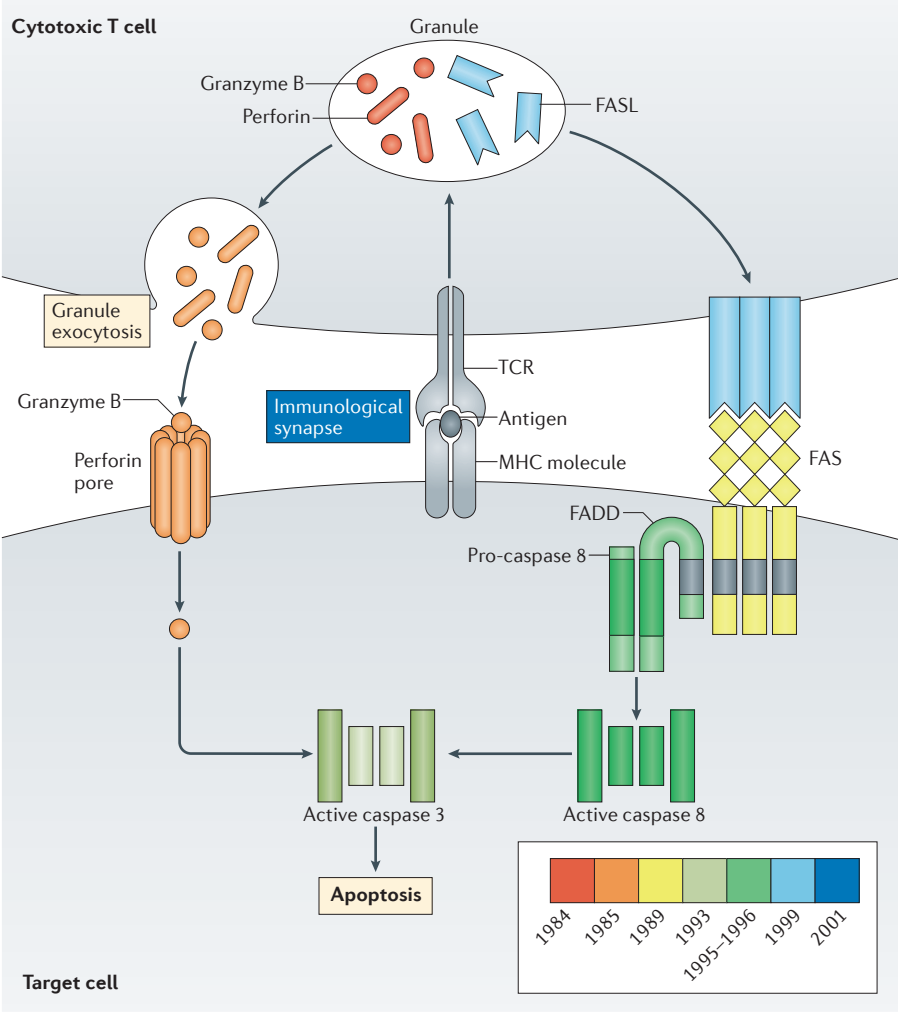


Fig 2



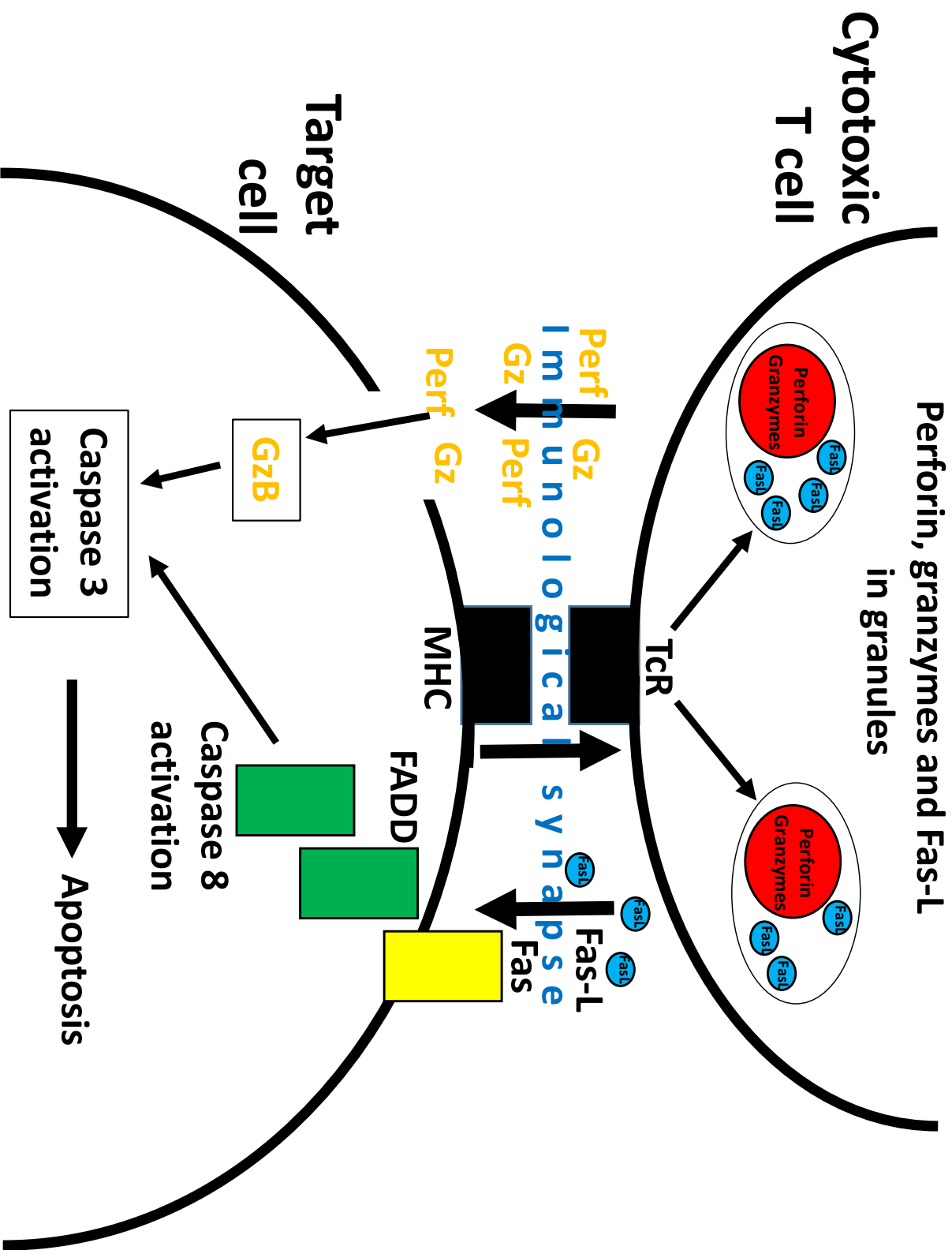
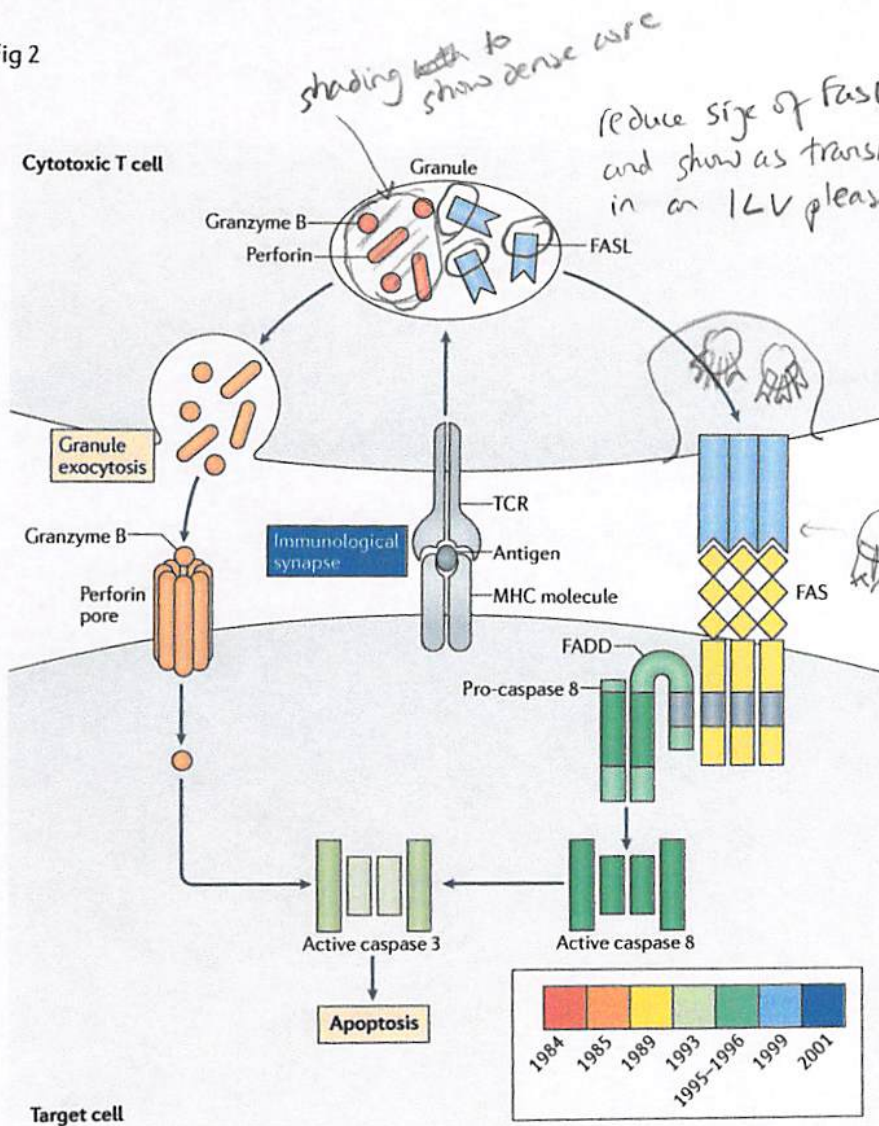


Figure 2



1984 1985 1989 1993 1995-2001 1996

Fig 2



shading both to show dense core
 reduce size of Fasl
 and show as transmembrane
 in an ILV please :-

It should also be shown
 as released on a vesicle
 with some granzyme and
 perforin

- ① Perforin & granzyme should be shown in a dense core, for which I suggest a shaded grey circle 
- ② FasL should be across the membranes of ILVs 
- ③ FasL should be shown as released on ILVs, so the size of the Fas receptor will need to be reduced accordingly
- ④ Really & truly the perforin/granzyme & FasL should be shown as released together