An early history of T cell-mediated cytotoxicity

Pierre Golstein and Gillian M. Griffiths

Pierre Golstein is at the Centre d'Immunologie de Marseille-Luminy, Marseille, France.

Gillian Griffiths is at the Cambridge Institute for Medical Research, Cambridge Biomedical Campus, Cambridge, UK.

e-mails: golstein@ciml.univ-mrs.fr; gg305@cam.ac.uk

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 REFS[1-3]).

[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 microscopically[1]. 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 death[2]. Also, lymph node or thoracic duct lymphoid cells from allosensitized rodents were able to cluster around and destroy donor kidney cells in culture[4].

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 plaques[7]. Cytotoxicity mediated by mouse alloimmune lymph node cells could be partially blocked by alloantibodies[8].

In a xenogeneic **[G]** combination, lymphoid cells from mice immunized with human HeLa cells were cytotoxic to monolayers of HeLa cells[9]. 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 mimick 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 vitro[12]. 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 cells[13].

[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 complement[16–18] 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 radiosiotopes 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 phosphatidylserine [21-23] 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 (REFS[25-27]), heterologous anti-T[28] 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 cytotoxicity[27], 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 antigen[34]. 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 surface[35], but could not retain antigen-specific T cells[36]. It was then realized that the recognition of antigen by T cells requires the presentation of this antigen through MHC molecules at the cell surface[37-40]; 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 antigens[41], 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 antigens[42]. 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 cells[28]. 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 sensitization[47]. 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 obtained[48] using tumour target cells and cytotoxic alloimmune lymphocytes from peritoneal exudate[49]. 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 cells[48]. 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 formation[51].

[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 lysis[52] and similar to the apoptotic cell death that had recently been described in detail[53]. 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 machinery [52]. 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 CTLs[54].

These and further more detailed phenomenological advances obtained through diverse analytical methods[55,56] were abundantly reviewed at the time[57-65]. An important development during this period was the ability to clone CTLs[66-68]. 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 granules[74-77] and was found to be homologous to the ninth component of complement[78]. 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 material[79] 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 CTLs[84-87] and moreover were localized in their granules[88-90], 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 factor[94] 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 experiments[96-98]. 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 roles[99-103].

[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 cytotoxic[104], which lead researchers to discuss alternative explanations for cytotoxicity including, in particular, cell surface death receptors[105]. 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+} (REFS[106-110]).

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 titles[111].

[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 interleukins[112], 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 loose 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 sensitive[114].

While these results were being obtained, a striking report by Shigekazu Nagata and colleagues[115] 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 FAS[114]. 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 cells[114]. 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 mechanism[118-121].

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

cells[122]. 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 homeostasis[124].

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 REFS[125-128]). 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 perforin[131], but also in other molecules[132], 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 Ca^{2+} upon target cell recognition[133]. Following early observations in CTLs and NK cells of polarization of the Golgi apparatus and the microtubule-organizing centre towards target cells[134-136], confocal imaging revealed the remarkable reorganization of receptors that occurs at the interface between T cells and antigen-presenting cells [137]. 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 cells[138]. 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 signalling[139] 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 BID [101]. Several granzymes could also lead to non-apoptotic forms of cell death[99]. Moreover, granzymes and granulysin could target and destroy intracellular parasites[141-144].

[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 membrane [145]. 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 cells[146]. Moreover, ubiquitylation signals in the cytosolic tail of FASL target it to intraluminal vesicles within such cytotoxic granules[147], which is consistent with the observation of FASL activity within vesicle preparations[148]. Thus, FASL is found in intraluminal vesicles that surround the perforin-containing dense core within cytotoxic granules[149]. 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 trimer[150], FAS was shown to signal to FAS-associated death domain protein (FADD)[151], leading to caspase 8 activation[152] then caspase 3 activation and apoptosis. FAS-induced signalling was shown to be regulated in part by molecular recruitment to membrane rafts[153] and by post-translational modifications[154]. FAS signalling also induced various functions in addition to cell death[155-158]. The lymphadenopathy that is prominent in mouse and human[159] 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 lymphocytes[163]. 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 identified[165]. *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 differentiation[170] and lupus-like autoimmune disease[171], which suggested that PD1 maintains peripheral self-tolerance by negatively regulating immune responses[172]. The different phenotypyes 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 subsets[173]. If CTLA4 and PD1 negatively regulate the activation of CTLs, contributing to their exhaustion[174], 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 underway [181]. 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 diseases[186] and against graft rejection[187,188]. A possible similar use of PD1 agonists is also being considered[189].

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 patient[190], 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 malignancies[193].

[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 is has progressed beyond basic science and is dominated by medical applications, hopefully leading to further exciting therapeutic advances.

REFERENCES

1 Govaerts, A. Cellular antibodies in kidney homotransplantation. *J. Immunol.* **85**, 516-522 (1960).

2 Rosenau, W. & Moon, H. D. Lysis of homologous cells by sensitized lymphocytes in tissue culture. *J. Natl Cancer Inst.* **27**, 471-483 (1961).

3 Amos, D. B. The use of simplified systems as an aid to the interpretation of mechanisms of graft rejection. *Prog. Allergy* **6**, 468-538 (1962).

4 Wilson, D. B. The Reaction of Immunologically Activated Lymphoid Cells against Homologous Lymphoid Cells against Homologous Target Tissue Cells in Vitro. *J. Cell. Comp. Physiol.* **62**, 273-286 (1963).

5 Brunner, K. T., Mauel, J. & Schindler, R. In vitro studies of cell-bound immunity ; cloning assay of the cytotoxic action of sensitized lymphoid cells on allogeneic target cells. *Immunology* **11**, 499-506 (1966).

6 Friedman, H. Inhibition of Antibody Plaque Formation by Sensitized Lymphoid Cells: Rapid Indicator of Transplantation Immunity. *Science* **145**, 607-609 (1964).

7 Granger, G. A. & Weiser, R. S. Homograft target cells : specific destruction in vitro by contact interaction with immune macrophages. *Science* **145**, 1427-1429 (1964).

8 Möller, E. Antagonistic effects of humoral isoantibodies on the in vitro cytotoxicity of immune lymphoid cells. *J. Exp. Med.* **122**, 11-23 (1965).

9 Stuart, A. E. The cytotoxic effect of heterologous lymphoid cells. *Lancet* **2**, 180-182 (1962).

10 Granger, G. A. & Williams, T. W. Lymphocyte cytotoxicity in vitro : activation and release of a cytotoxic factor. *Nature* **218**, 1253-1254 (1968).

11 Ruddle, N. H. & Waksman, B. H. Cytotoxicity mediated by soluble antigen and lymphocytes in delayed hypersensitivity. III. Analysis of mechanism. *J. Exp. Med.* **128**, 1267-1279 (1968).

12 Koprowski, H. & Fernandes, M. V. Autosensitization reaction in vitro. Contactual agglutination of sensitized lymph node cells in brain tissue culture accompanied by destruction of glial elements. *J. Exp. Med.* **116**, 467-476 (1962).

13 Berg, O. & Kallen, B. White blood cells from animals with experimental allergic encephalomyelitis tested on glia cells in tissue culture. *Acta Pathol. Microbiol. Scand.* **58**, 33-42 (1963).

14 Perlmann, P. & Broberger, O. In vitro studies of ulcerative colitis. II. Cytotoxic action of white blood cells from patients on human fetal colon cells. *J. Exp. Med.* **117**, 717-733 (1963).

15 Vainio, T., Koskimies, O., Perlmann, P., Perlmann, H. & Klein, G. In vitro cytotoxic effect of lymphoid cells from mice immunized with allogeneic tissue. *Nature* **204**, 453-455 (1964).

16 Sanderson, A. R. Cytotoxic Reactions of Mouse Iso-Antisera: Preliminary Considerations. *Br. J. Exp. Pathol.* **45**, 398-408 (1964).

17 Sanderson, A. R. Applications of Iso-Immune Cytolysis Using Radiolabelled Target Cells. *Nature* **204**, 250-253 (1964).

18 Wigzell, H. Quantitative Titrations of Mouse H-2 Antibodies Using Cr-51-Labelled Target Cells. *Transplantation* **3**, 423-431 (1965).

19 Holm, G. & Perlmann, P. Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. *Immunology* **12**, 525-536 (1967).

20 Brunner, K. T., Mauel, J., Cerottini, J.-C. & Chapuis, B. Quantitative assay of the lytic action of immune lymphoid cells on 51Cr-labelled allogeneic target cells in vitro ; inhibition by isoantibody and by drugs. *Immunology* **14**, 181-196 (1968).

21 Henry, C. M., Hollville, E. & Martin, S. J. Measuring apoptosis by microscopy and flow cytometry. *Methods* **61**, 90-97 (2013).

22 Frick, M. *et al.* Distinct patterns of cytolytic T-cell activation by different tumour cells revealed by Ca2+ signalling and granule mobilization. *Immunology* **150**, 199-212 (2017).

23 Vanden Berghe, T. *et al.* Determination of apoptotic and necrotic cell death in vitro and in vivo. *Methods* **61**, 117-129 (2013).

24 Perlmann, P. & Holm, G. Cytotoxic effects of lymphoid cells in vitro. *Adv.Immunol.* **11**, 117-193 (1969).

25 Cerottini, J.-C., Nordin, A. A. & Brunner, K. T. Specific in vitro cytotoxicity of thymus-derived lymphocytes sensitized to alloantigens. *Nature* **228**, 1308-1309 (1970).

26 Lonai, P., Clark, W. R. & Feldman, M. Participation of theta-bearing cell in an in vitro assay of transplantation immunity. *Nature* **229**, 566-567 (1971).

27 Golstein, P. & Blomgren, H. Further evidence for autonomy of T cells mediating specific in vitro cytotoxicity : efficiency of very small amounts of highly purified T cells. *Cell. Immunol.* **9**, 127-141 (1973).

28 Golstein, P., Wigzell, H., Blomgren, H. & Svedmyr, E. A. Cells mediating specific in vitro cytotoxicity. II. Probable autonomy of thymus-processed lymphocytes (T cells) for the killing of allogeneic target cells. *J. Exp. Med.* **135**, 890-906 (1972).

29 Cantor, H. & Boyse, E. A. Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141**, 1376-1389 (1975).

30 Golstein, P., Wigzell, H., Blomgren, H. & Svedmyr, E. A. J. Autonomy of thymus-processed lymphocytes (T cells) for their education into cytotoxic T cells. *Eur. J. Immunol.* **2**, 498-501 (1972).

31 Sprent, J. & Miller, J. F. Activation of thymus cells by histocompatibility antigens. *Nat. New Biol.* **234**, 195-198 (1971).

32 Blomgren, H. & Svedmyr, E. In vitro stimulation of mouse thymus cells by PHA and allogeneic cells. *Cell. Immunol.* **2**, 285-299 (1971).

33 Lohmann-Matthes, M. L. & Fischer, H. Specific cytotoxicity of a mouse thymocyte population sensitized in vitro against H-2 alloantigens. *Eur. J. Immunol.* **2**, 290-292 (1972).

34 Crone, M., Koch, C. & Simonsen, M. The elusive T cell receptor. *Transplant. Rev.* **10**, 36-56 (1972).

35 Wigzell, H. & Andersson, B. Cell separation on antigen-coated columns. Elimination of high rate antibody-forming cells and immunological memory cells. *J. Exp. Med.* **129**, 23-36 (1969).

36 Wigzell, H. Specific fractionation of immunocompetent cells. *Transplant. Rev.* **5**, 76-104 (1970).

37 Kindred, B. & Shreffler, D. C. H-2 dependence of co-operation between T and B cells in vivo. *J. Immunol.* **109**, 940-943 (1972).

38 Katz, D. H., Hamaoka, T., Dorf, M. E. & Benacerraf, B. Cell interactions between histoincompatible T and B lymphocytes. The H-2 gene complex determines successful physiologic lymphocyte interactions. *Proc. Natl Acad. Sci. U S A.* **70**, 2624-2628 (1973).

39 Shearer, G. M. Cell-mediated cytotoxicity to trinitrophenyl-modified syngeneic lymphocytes. *Eur. J. Immunol.* **4**, 527-533 (1974).

40 Zinkernagel, R. M. & Doherty, P. C. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702 (1974).

41 Brondz, B. D. Interaction of immune lymphocytes in vitro with normal and neoplastic tissue cells. *Folia Biol.* **10**, 164-175 (1964).

42 Brondz, B. D. Complex specificity of immune lymphocytes in allogeneic cell cultures. *Folia Biol.* **14**, 115-131 (1968).

43 Brondz, B. D. & Snegirova, A. E. Interaction of immune lymphocytes with the mixtures of target cells possessing selected specificities of the H-2 immunizing allele. *Immunology* **20**, 457-468 (1971).

44 Brondz, B. D. Lymphocyte receptors and mechanisms of in vitro cell-mediated immune reactions. *Transplant. Rev.* **10**, 112-151 (1972).

45 Golstein, P., Svedmyr, E. A. J. & Wigzell, H. Cells mediating specific in vitro cytotoxicity. I. Detection of receptor-bearing lymphocytes. *J. Exp. Med.* **134**, 1385-1402 (1971).

46 Berke, G. & Levey, R. H. Cellular immunoabsorbents in transplantation immunity. Specific in vitro deletion and recovery of mouse lymphoid cells sensitized against allogeneic tumors. *J. Exp. Med.* **135**, 972-984 (1972).

47 Altman, A., Cohen, I. R. & Feldman, M. Normal T-cell receptors for alloantigens. *Cell. Immunol.* **7**, 134-142 (1973).

48 Zagury, D., Bernard, J., Thiernesse, N., Feldman, M. & Berke, G. Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes : conjugation, killing and recycling at the single cell level. *Eur. J. Immunol.* **5**, 818-822 (1975).

49 Berke, G., Sullivan, K. A. & Amos, B. Rejection of ascites tumor allografts. I. Isolation, characterization, and in vitro reactivity of peritoneal lymphoid effector cells from BALB/c mice immune to EL4 leukosis. *J. Exp. Med.* **135**, 1334-1350 (1972).

50 Ax, W., Malchow, H., Zeiss, I. & Fischer, H. The behaviour of lymphocytes in the process of target cell destruction in vitro. *Exp. Cell Res.* **53**, 108-116 (1968).

51 Koren, H. S., Ax, W. & Freund-Moelbert, E. Morphological observations on the contact-induced lysis of target cells. *Eur. J. Immunol.* **3**, 32-37 (1973).

52 Sanderson, C. J. The mechanism of T cell mediated cytotoxicity. II. Morphological studies of cell death by time-lapse microcinematography. *Proc. R. Soc. Lond. B Biol. Sci.* **192**, 241-255 (1976).

53 Kerr, J. F. R., Wyllie, A. H. & Currie, A. R. Apoptosis : a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Brit. J. Cancer* **26**, 239-257 (1972).

54 Golstein, P. Sensitivity of cytotoxic T cells to T cell-mediated cytotoxicity. *Nature* **252**, 81-83 (1974).

55 Wagner, H. & Röllinghoff, M. T cell-mediated cytotoxicity : Discrimination between antigen recognition, lethal hit and cytolysis phase. *Eur. J. Immunol.* **4**, 745-750 (1974).

56 Gately, M. K. & Martz, E. Early steps in specific tumor cell lysis by sensitized mouse T lymphocytes. III. Resolution of two distinct roles for calcium in the cytolytic process. *J. Immunol.* **122**, 482-489 (1979).

57 Cerottini, J.-C. & Brunner, K. T. Cell-mediated cytotoxicity, allograft rejection, and tumor immunity. *Adv.Immunol.* **18**, 67-132 (1974).

58 Henney, C. S. T-cell-mediated cytolysis : an overview of some current issues. *Contemp. Top. Immunobiol.* **7**, 245-272 (1977).

59 Martz, E. Mechanism of specific tumor-cell lysis by alloimmune T lymphocytes : resolution and characterization of discrete steps in the cellular interaction. *Contemp. Top. Immunobiol.* **7**, 301-361 (1977).

60 Golstein, P. & Smith, E. T. Mechanism of T-cell-mediated cytolysis: the lethal hit stage. *Contemp. Top. Immunobiol.* **7**, 273-300 (1977).

61 Sanderson, C. J. The mechanism of lymphocyte-mediated cytotoxicity. *Biol. Rev. Camb. Philos. Soc.* **56**, 153-197 (1981).

62 Bonavida, B. *et al.* Molecular interactions in T-cell-mediated cytotoxicity. *Immunol. Rev.* **72**, 119-141 (1983).

63 Russell, J. H. Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* **72**, 97-118 (1983).

64 Martz, E., Heagy, W. & Gromkowski, S. H. The mechanism of CTL-mediated killing: monoclonal antibody analysis of the roles of killer and target-cell membrane proteins. *Immunol. Rev.* **72**, 73-96 (1983).

65 Berke, G. Cytotoxic T-lymphocytes. How do they function? *Immunol. Rev.* **72**, 5-42 (1983).

66 Baker, P. E., Gillis, S. & Smith, K. A. Monoclonal cytolytic T-cell lines. *J. Exp. Med.* **149**, 273-278 (1979).

67 Nabholz, M. *et al.* Established murine cytolytic T-cell lines as tools for a somatic cell genetic analysis of T-cell functions. *Immunol. Rev.* **51**, 125-156 (1980).

68 Albert, F., Buferne, M., Boyer, C. & Schmitt-Verhulst, A.-M. Interactions between MHC-encoded products and cloned T cells.I. Fine specificity for induction of proliferation and lysis. *Immunogenetics* **16**, 533-549 (1982).

69 Dourmashkin, R. R., Deteix, P., Simone, C. B. & Henkart, P. Electron microscopic demonstration of lesions in target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin. Exp. Immunol.* **42**, 554-560 (1980).

70 Dennert, G. & Podack, E. R. Cytolysis by H-2-specific T killer cells. Assembly of tubular complexes on target membranes. *J. Exp. Med.* **157**, 1483-1495 (1983).

71 Criado, M., Lindstrom, J. M., Anderson, C. G. & Dennert, G. Cytotoxic granules from killer cells: specificity of granules and insertion of channels of defined size into target membranes. *J. Immunol.* **135**, 4245-4251 (1985).

72 Podack, E. R. & Konigsberg, P. J. Cytolytic T cell granules. Isolation, structural, biochemical, and functional characterization. *J. Exp. Med.* **160**, 695-710 (1984).

73 Henkart, P. A., Millard, P. J., Reynolds, C. W. & Henkart, M. P. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J. Exp. Med.* **160**, 75-93 (1984).

74 Podack, E. R., Young, J. D.-E. & Cohn, Z. A. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. *Proc. Natl Acad. Sci. U S A.* **82**, 8629-8633 (1985).

75 Henkart, P. A. Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **3**, 31-58 (1985).

76 Masson, D. & Tschopp, J. Isolation of a lytic, pore-forming protein (perforin) from cytolytic T lymphocytes. *J. Biol. Chem.* **260**, 9069-9072 (1985).

77 Young, J. D.-E., Hengartner, H., Podack, E. R. & Cohn, Z. A. Purification and characterization of a cytolytic pore-forming protein from granules of cloned lymphocytes with natural killer activity. *Cell* **44**, 849-859 (1986).

78 Shinkai, Y., Takio, K. & Okumura, K. Homology of perforin to the ninth component of complement (C9). *Nature* **334**, 525-527 (1988).

79 Acha-Orbea, H., Scarpellino, L., Hertig, S., Dupuis, M. & Tschopp, J. Inhibition of lymphocyte mediated cytotoxicity by perforin antisense oligonucleotides. *EMBO J.* **9**, 3815-3819 (1990).

80 Kägi, D. *et al.* Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature* **369**, 31-37 (1994).

81 Lowin, B., Beermann, F., Schmidt, A. & Tschopp, J. A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity. *Proc. Natl Acad. Sci. U S A.* **91**, 11571-11575 (1994).

82 Chang, T. W. & Eisen, H. N. Effects of N-«-tosyl-L-lysyl-chloromethylketone on the activity of cytotoxic T lymphocytes. *J. Immunol.* **124**, 1028-1033 (1980).

83 Redelman, D. & Hudig, D. The mechanism of cell-mediated cytotoxicity.I. Killing by murine cytotoxic T lymphocytes requires cell surface thiols and activated proteases. *J. Immunol.* **124**, 870-878 (1980).

84 Pasternack, M. S. & Eisen, H. N. A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature* **314**, 743-745 (1985).

85 Pasternack, M. S., Verret, C. R., Liu, M. A. & Eisen, H. N. Serine esterase in cytolytic T lymphocytes. *Nature* **322**, 740-743 (1986).

86 Kramer, M. D. *et al.* Characterization and isolation of a trypsin-like serine protease from a long-term culture cytolytic T cell line and its expression by functionally distinct T cells. *J. Immunol.* **136**, 4644-4651 (1986).

87 Simon, M. M., Hoschützky, H., Fruth, U., Simon, H.-G. & Kramer, M. D. Purification and characterization of a T cell specific serine proteinase (TSP-1) from cloned cytolytic T lymphocytes. *EMBO J.* **5**, 3267-3274 (1986).

88 Young, J. D.-E. *et al.* Isolation and characterization of a serine esterase from cytolytic T cell granules. *Cell* **47**, 183-194 (1986).

89 Masson, D., Nabholz, M., Estrade, C. & Tschopp, J. Granules of cytolytic T-lymphocytes contain two serine esterases. *EMBO J.* **5**, 1595-1600 (1986).

90 Masson, D. & Tschopp, J. A family of serine esterases in lytic granules of cytolytic T lymphocytes. *Cell* **49**, 679-685 (1987).

91 Brunet, J.-F., Denizot, F. & Golstein, P. A differential molecular biology search for genes preferentially expressed in functional T lymphocytes : the CTLA genes. *Immunol. Rev.* **103**, 21-36 (1988).

92 Brunet, J.-F. *et al.* The inducible cytotoxic-T-lymphocyte-associated gene transcript CTLA-1 sequence and gene localization to mouse chromosome 14. *Nature* **322**, 268-271 (1986).

93 Brunet, J.-F. *et al.* CTLA-1 and CTLA-3 serine-esterase transcripts are detected mostly in cytotoxic cells, but not only and not always. *J.Immunol.* **138**, 4102-4105 (1987).

94 Gershenfeld, H. K. & Weissman, I. L. Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte. *Science* **232**, 854-858 (1986).

95 Lobe, C. G., Finlay, B. B., Paranchych, W., Paetkau, V. H. & Bleackley, R. C. Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science* **232**, 858-861 (1986).

96 Shiver, J. W., Su, L. & Henkart, P. A. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell* **71**, 315-322 (1992).

97 Shi, L., Kam, C.-M., Powers, J. C., Aebersold, R. & Greenberg, A. H. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. *J. Exp. Med.* **176**, 1521-1529 (1992).

98 Shi, L., Kraut, R. P., Aebersold, R. & Greenberg, A. H. A natural killer cell granule protein that induces DNA fragmentation and apoptosis. *J. Exp. Med.* **175**, 553-566 (1992).

99 Chowdhury, D. & Lieberman, J. Death by a thousand cuts: granzyme pathways of programmed cell death. *Annu. Rev. Immunol.* **26**, 389-420 (2008).

100 Ewen, C. L., Kane, K. P. & Bleackley, R. C. A quarter century of granzymes. *Cell Death Differ.* **19**, 28-35 (2012).

101 Voskoboinik, I., Whisstock, J. C. & Trapani, J. A. Perforin and granzymes: function, dysfunction and human pathology. *Nat. Rev. Immuno.l* **15**, 388-400 (2015).

102 Dotiwala, F. *et al.* Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites. *Nat. Med.* **22**, 210-216 (2016).

103 Chiusolo, V. *et al.* Granzyme B enters the mitochondria in a Sam50-, Tim22- and mtHsp70-dependent manner to induce apoptosis. *Cell Death Differ.* **24**, 747-758 (2017).

104 Berke, G. The cytolytic T lymphocyte and its mode of action. *Immunol.Lett.* **20**, 169-178 (1989).

105 Berke, G. T-cell-mediated cytotoxicity. *Curr. Opin. Immunol.* **3**, 320-325 (1991).

106 MacLennan, I. C. M., Gotch, F. M. & Golstein, P. Limited specific T-cell mediated cytolysis in the absence of extracellular Ca++. *Immunology* **39**, 109-117 (1980).

107 Tirosh, R. & Berke, G. T lymphocyte-mediated cytolysis as an excitatory process of the target. I. Evidence that the target may be the site of Ca++ action. *Cell. Immunol.* **95**, 113-123 (1985).

108 Trenn, G., Takayama, H. & Sitkovsky, M. V. Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T-lymphocytes. *Nature* **330**, 72-74 (1987).

109 Ostergaard, H. L., Kane, K. P., Mescher, M. F. & Clark, W. R. Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature* **330**, 71-72 (1987).

110 Young, J. D.-E., Clark, W. R., Liu, C.-C. & Cohn, Z. A. A calcium- and perforin-independent pathway of killing mediated by murine cytolytic lymphocytes. *J. Exp. Med.* **166**, 1894-1899 (1987).

111 Golstein, P. Cytotoxic-T-cell melodrama. *Nature* **327**, 12 (1987).

112 Conzelmann, A., Corthésy, P., Cianfriglia, M., Silva, A. & Nabholz, M. Hybrids between rat lymphoma and mouse T cells with inducible cytolytic activity. *Nature* **298**, 170-172 (1982).

113 Golstein, P., Mattéi, M.-G., Foa, C. & Luciani, M.-F. in *Apoptosis and the Immune Response* (ed C.D. Gregory) 143-168 (John Wiley and Sons, Inc., 1995).

114 Rouvier, E., Luciani, M.-F. & Golstein, P. Fas involvement in Ca^{++}-independent T cell-mediated cytotoxicity. *J. Exp. Med.* **177**, 195-200 (1993).

115 Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. & Nagata, S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**, 314-317 (1992).

116 Yonehara, S., Ishii, A. & Yonehara, M. A cell-killing monoclonal antibody (Anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169**, 1747-1756 (1989).

117 Trauth, B. C. *et al.* Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* **245**, 301-305 (1989).

118 Kägi, D. *et al.* Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science* **265**, 528-530 (1994).

119 Lowin, B., Hahne, M., Mattmann, C. & Tschopp, J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature* **370**, 650-652 (1994).

120 Walsh, C. M. *et al.* Immune function in mice lacking the perforin gene. *Proc. Natl Acad. Sci. U S A.* **91**, 10854-10858 (1994).

121 Kojima, H. *et al.* Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity.* **1**, 357-364 (1994).

122 Suda, T., Takahashi, T., Golstein, P. & Nagata, S. Molecular cloning and expression of the Fas ligand : a novel member of the tumor necrosis factor family. *Cell* **75**, 1169-1178 (1993).

123 Nagata, S. & Golstein, P. The Fas death factor. *Science* **267**, 1449-1456 (1995).

124 Krammer, P. H. CD95's deadly mission in the immune system. *Nature* **407**, 789-795 (2000).

125 Glimcher, L. H., Townsend, M. J., Sullivan, B. M. & Lord, G. M. Recent developments in the transcriptional regulation of cytolytic effector cells. *Nat. Rev. Immunol.* **4**, 900-911 (2004).

126 Man, K. & Kallies, A. Synchronizing transcriptional control of T cell metabolism and function. *Nat. Rev. Immunol.* **15**, 574-584 (2015).

127 Xin, A. *et al.* A molecular threshold for effector CD8(+) T cell differentiation controlled by transcription factors Blimp-1 and T-bet. *Nat. Immunol.* **17**, 422-432 (2016).

128 Ahrends, T. *et al.* CD4+ T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity* **47**, 848-861 e845 (2017).

129 Burkhardt, J. K., Hester, S., Lapham, C. K. & Argon, Y. The lytic granules of natural killer cells are dual-function organelles combining secretory and pre-lysosomal compartments. *J. Cell Biol.* **111**, 2327-2340 (1990).

130 Peters, P. J. *et al.* Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J. Exp. Med.* **173**, 1099-1109 (1991).

131 Stepp, S. E. *et al.* Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* **286**, 1957-1959 (1999).

132 Pachlopnik Schmid, J. *et al.* Inherited defects in lymphocyte cytotoxic activity. *Immunol. Rev.* **235**, 10-23, doi:IMR890 (2010).

133 Poenie, M., Tsien, R. Y. & Schmitt-Verhulst, A.-M. Sequential activation and lethal hit measured by (Ca++)i in individual cytolytic T cells and targets. *EMBO J.* **6**, 2223-2232 (1987).

134 Bykovskaya, S. N., Rytenko, A. N., Rauschenbach, M. O. & Bykovsky, A. F. Ultrastructural alteration of cytolytic T lymphocytes following their interaction with target cells. I. Hypertrophy and change of orientation of the Golgi apparatus. *Cell. Immunol.* **40**, 164-174 (1978).

135 Geiger, B., Rosen, D. & Berke, G. Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J. Cell Biol.* **95**, 137-143 (1982).

136 Kupfer, A., Dennert, G. & Singer, S. J. Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. *Proc. Natl Acad. Sci. U S A.* **80**, 7224-7228 (1983).

137 Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**, 82-86 (1998).

138 Stinchcombe, J. C., Bossi, G., Booth, S. & Griffiths, G. M. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751-761 (2001).

139 Stinchcombe, J. C., Majorovits, E., Bossi, G., Fuller, S. & Griffiths, G. M. Centrosome polarization delivers secretory granules to the immunological synapse. *Nature* **443**, 462-465 (2006).

140 Lopez, J. A. *et al.* Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. *Blood* **121**, 2659-2668 (2013).

141 Stenger, S. *et al.* An antimicrobial activity of cytolytic T cells mediated by granulysin. *Science* **282**, 121-125 (1998).

142 Ochoa, M. T. *et al.* T-cell release of granulysin contributes to host defense in leprosy. *Nat. Med.* **7**, 174-179 (2001).

143 Stegelmann, F. *et al.* Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against Mycobacterium tuberculosis. *J. Immunol.* **175**, 7474-7483 (2005).

144 Dotiwala, F. *et al.* Granzyme B Disrupts Central Metabolism and Protein Synthesis in Bacteria to Promote an Immune Cell Death Program. *Cell* **171**, 1125-1137 e1111 (2017).

145 Tanaka, M., Itai, T., Adachi, M. & Nagata, S. Downregulation of Fas ligand by shedding. *Nat.Med.* **4**, 31-36 (1998).

146 Bossi, G. & Griffiths, G. M. Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. *Nat.Med.* **5**, 90-96 (1999).

147 Zuccato, E. *et al.* Sorting of Fas ligand to secretory lysosomes is regulated by mono-ubiquitylation and phosphorylation. *J. Cell Sci.* **120**, 191-199 (2007).

148 Martinez-Lorenzo, M. J. *et al.* Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. *J. Immunol.* **163**, 1274-1281 (1999).

149 Lee, J., Dieckmann, N. M., Edgar, J., Griffiths, G. M. & Siegel, R. M. Fas Ligand localizes to intraluminal vesicles within NK cell cytolytic granules, delivering membrane-bound FasL to the immune synapse. *Immunity, Inflammation and Disease.* **in press** (2018).

150 Schneider, P. *et al.* Characterization of Fas (Apo-1, CD95)-Fas ligand interaction. *J.Biol. Chem.* **272**, 18827-18833 (1997).

151 Boldin, M. P. *et al.* A novel protein that interacts with the death domains of Fas/APO1 contains a sequence motif related to the death domain. *J. Biol. Chem.* **270**, 7795-7798 (1995).

152 Muzio, M. *et al.* FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85**, 817-827 (1996).

153 Hueber, A. O., Bernard, A. M., Herincs, Z., Couzinet, A. & He, H. T. An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. *EMBO Rep.* **3**, 190-196 (2002).

154 Rossin, A. *et al.* Fas palmitoylation by the palmitoyl acyltransferase DHHC7 regulates Fas stability. *Cell Death Differ.* **22**, 643-653 (2015).

155 Desbarats, J. & Newell, M. K. Fas engagement accelerates liver regeneration after partial hepatectomy. *Nat.Med.* **6**, 920-923 (2000).

156 Peter, M. E. *et al.* The CD95 receptor: apoptosis revisited. *Cell* **129**, 447-450 (2007).

157 Yamada, A., Arakaki, R., Saito, M., Kudo, Y. & Ishimaru, N. Dual Role of Fas/FasL-Mediated Signal in Peripheral Immune Tolerance. *Front. Immunol.* **8**, 403 (2017).

158 Le Gallo, M., Poissonnier, A., Blanco, P. & Legembre, P. CD95/Fas, Non-Apoptotic Signaling Pathways, and Kinases. *Front. Immunol.* **8**, 1216 (2017).

159 Rieux-Laucat, F. *et al.* Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* **268**, 1347-1349 (1995).

160 Balomenos, D., Shokri, R., Daszkiewicz, L., Vazquez-Mateo, C. & Martinez, A. C. On How Fas Apoptosis-Independent Pathways Drive T Cell Hyperproliferation and Lymphadenopathy in lpr Mice. *Front. Immunol.* **8**, 237 (2017).

161 Van den Broek, M. F. *et al.* Decreased tumor surveillance in perforin-deficient mice. *J. Exp. Med.* **184**, 1781-1790 (1996).

162 Smyth, M. J. *et al.* Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J. Exp. Med.* **192**, 755-760 (2000).

163 Rosenberg, S. A. & Lotze, M. T. Cancer immunotherapy using interleukin-2 and interleukin-2-activated lymphocytes. *Annu. Rev. Immunol.* **4**, 681-709 (1986).

164 Brunet, J.-F. *et al.* A new member of the immunoglobulin superfamily - CTLA-4. *Nature* **328**, 267-270 (1987).

165 Ishida, Y., Agata, Y., Shibahara, K. & Honjo, T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* **11**, 3887-3895 (1992).

166 Waterhouse, P. *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* **270**, 985-988 (1995).

167 Tivol, E. A. *et al.* Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* **3**, 541-547 (1995).

168 Kuehn, H. S. *et al.* Immune dysregulation in human subjects with heterozygous germline mutations in CTLA4. *Science* **345**, 1623-1627 (2014).

169 Schubert, D. *et al.* Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. *Nat. Med.* **20**, 1410-1416 (2014).

170 Nishimura, H., Minato, N., Nakano, T. & Honjo, T. Immunological studies on PD-1 deficient mice: implication of PD-1 as a negative regulator for B cell responses. *Int. Immunol.* **10**, 1563-1572 (1998).

171 Nishimura, H., Nose, M., Hiai, H., Minato, N. & Honjo, T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* **11**, 141-151 (1999).

172 Nishimura, H. & Honjo, T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol.* **22**, 265-268 (2001).

173 Wei, S. C. *et al.* Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade. *Cell* **170**, 1120-1133 (2017).

174 Wherry, E. J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* **15**, 486-499 (2015).

175 Leach, D. R., Krummel, M. F. & Allison, J. P. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* **271**, 1734-1736 (1996).

176 Iwai, Y. *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl Acad. Sci. U S A.* **99**, 12293-12297 (2002).

177 Blank, C. *et al.* PD-L1/B7H-1 inhibits the effector phase of tumor rejection by T cell receptor (TCR) transgenic CD8+ T cells. *Cancer Res.* **64**, 1140-1145 (2004).

178 Hodi, F. S. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med.* **363**, 711-723 (2010).

179 Topalian, S. L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366**, 2443-2454 (2012).

180 Hamid, O. *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N. Engl. J. Med.* **369**, 134-144 (2013).

181 Sharpe, A. H. Introduction to checkpoint inhibitors and cancer immunotherapy. *Immunol. Rev.* **276**, 5-8 (2017).

182 Wykes, M. N. & Lewin, S. R. Immune checkpoint blockade in infectious diseases. *Nat. Rev. Immunol.* **18**, 91-104 (2018).

183 Dyck, L. & Mills, K. H. G. Immune checkpoints and their inhibition in cancer and infectious diseases. *Eur. J. Immunol.* **47**, 765-779 (2017).

184 Linsley, P. S. *et al.* CTLA-4 is a second receptor for the B cell activation antigen B7. *J. Exp. Med.* **174**, 561-569 (1991).

185 Adams, A. B., Ford, M. L. & Larsen, C. P. Costimulation Blockade in Autoimmunity and Transplantation: The CD28 Pathway. *J. Immunol.* **197**, 2045-2050 (2016).

186 Ceeraz, S., Nowak, E. C., Burns, C. M. & Noelle, R. J. Immune checkpoint receptors in regulating immune reactivity in rheumatic disease. *Arthritis Res. Ther.* **16**, 469 (2014).

187 Lin, H. *et al.* Review of CTLA4Ig use for allograft immunosuppression. *Transplant.Proc.* **26**, 3200-3201 (1994).

188 Huber, M., Kemmner, S., Renders, L. & Heemann, U. Should belatacept be the centrepiece of renal transplantation? *Nephrol. Dial. Transplant.* **31**, 1995-2002 (2016).

189 Sandigursky, S., Silverman, G. J. & Mor, A. Targeting the programmed cell death-1 pathway in rheumatoid arthritis. *Autoimmun. Rev.* **16**, 767-773 (2017).

190 June, C. H. Adoptive T cell therapy for cancer in the clinic. *J. Clin. Invest.* **117**, 1466-1476 (2007).

191 Gross, G., Waks, T. & Eshhar, Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl Acad. Sci. U S A.* **86**, 10024-10028 (1989).

192 Eshhar, Z., Waks, T., Gross, G. & Schindler, D. G. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. *Proc. Natl Acad. Sci. U S A.* **90**, 720-724 (1993).

193 Gross, G. & Eshhar, Z. Therapeutic Potential of T Cell Chimeric Antigen Receptors (CARs) in Cancer Treatment: Counteracting Off-Tumor Toxicities for Safe CAR T Cell Therapy. *Annu. Rev. Pharmacol. Toxicol.* **56**, 59-83 (2016).

194 Schmidt, H. et al. Effector granules in human T lymphocytes: proteomic evidence for two distinct species of cytotoxic effector vesicles. *J. Proteome Res.* **10**, 1603-1620 (2011).

Acknowledgements

P.G. thanks Association pour la Recherche sur le Cancer for support, and Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, and Aix Marseille University for institutional support to the CIML. G.M.G. thanks the Wellcome Trust for research funding (grants 103930 and 100140). The authors thank all past and present members of their laboratories for their contribution to this research.

Author contributions

Both authors researched data for the article, discussed its content, and wrote, reviewed and edited the manuscript before submission.

Competing interests statement

The authors declare no competing interests.

Publisher's note

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 granzymes[149]. Alternatively, other studies have suggested that FASL is contained in distinct granules **for** perforin and granzymes[194]. 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
[URI /631/250/1619/554/1834/1269]
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.

Author notes

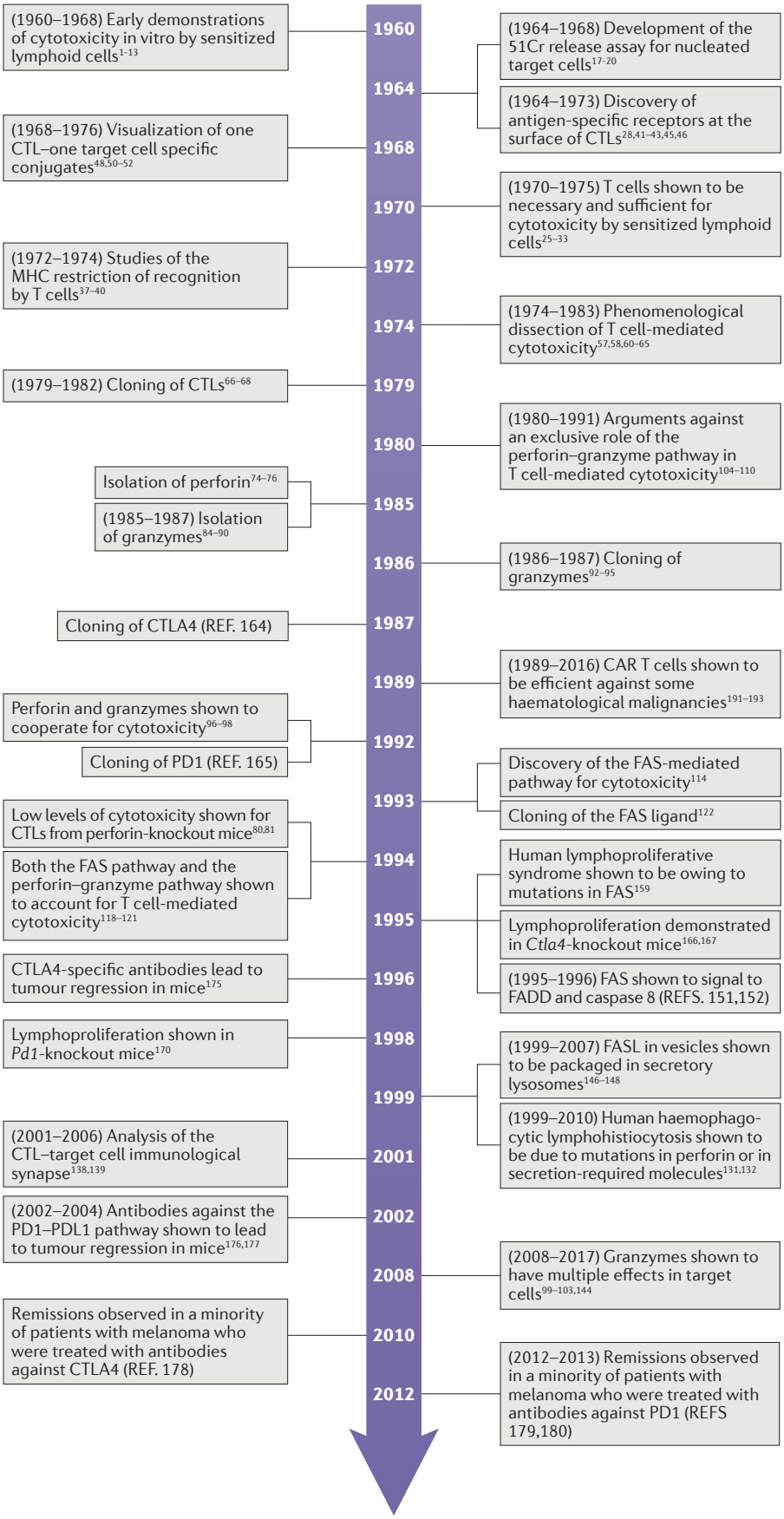
Please check these figures carefully and return any comments/amendments that you might have to me as soon as possible. In particular, we would like you to check the following:

- Do the figures convey the intended message?
- Are all the labels accurate and in the right place?
- Are all the arrows in the right place?
- Are any chemical structures correct?
- Have shapes and colours been used consistently and accurately throughout the figures?
- Have any of the figures been previously published, or have they been supplied by a colleague(s) who is not a named author on the article?

To mark up any corrections, please use the commenting tools in the PDF, or print and draw by hand, rather than directly editing the PDFs.

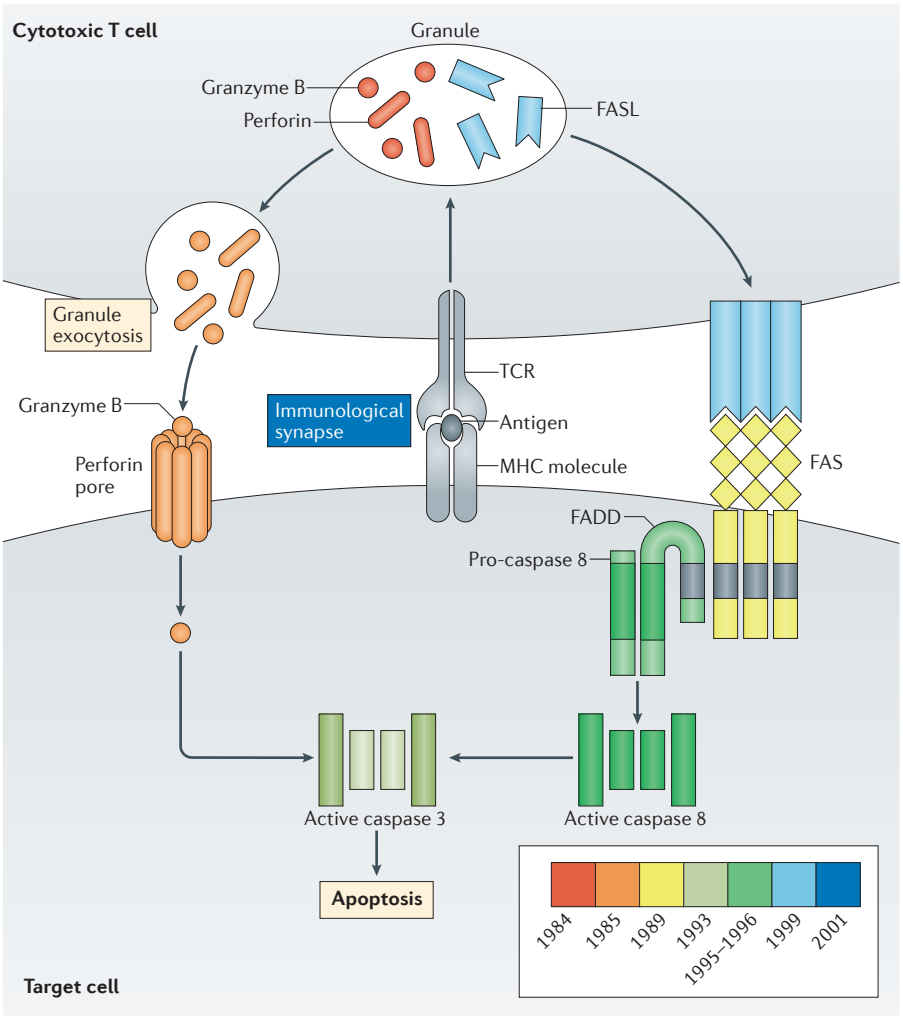
Manuscript number NRI-17-148 Griffiths 06|03|18

Fig 1



Manuscript number NRI-17-148 Griffiths 06|03|18

Fig 2



Nature Reviews | Immunology

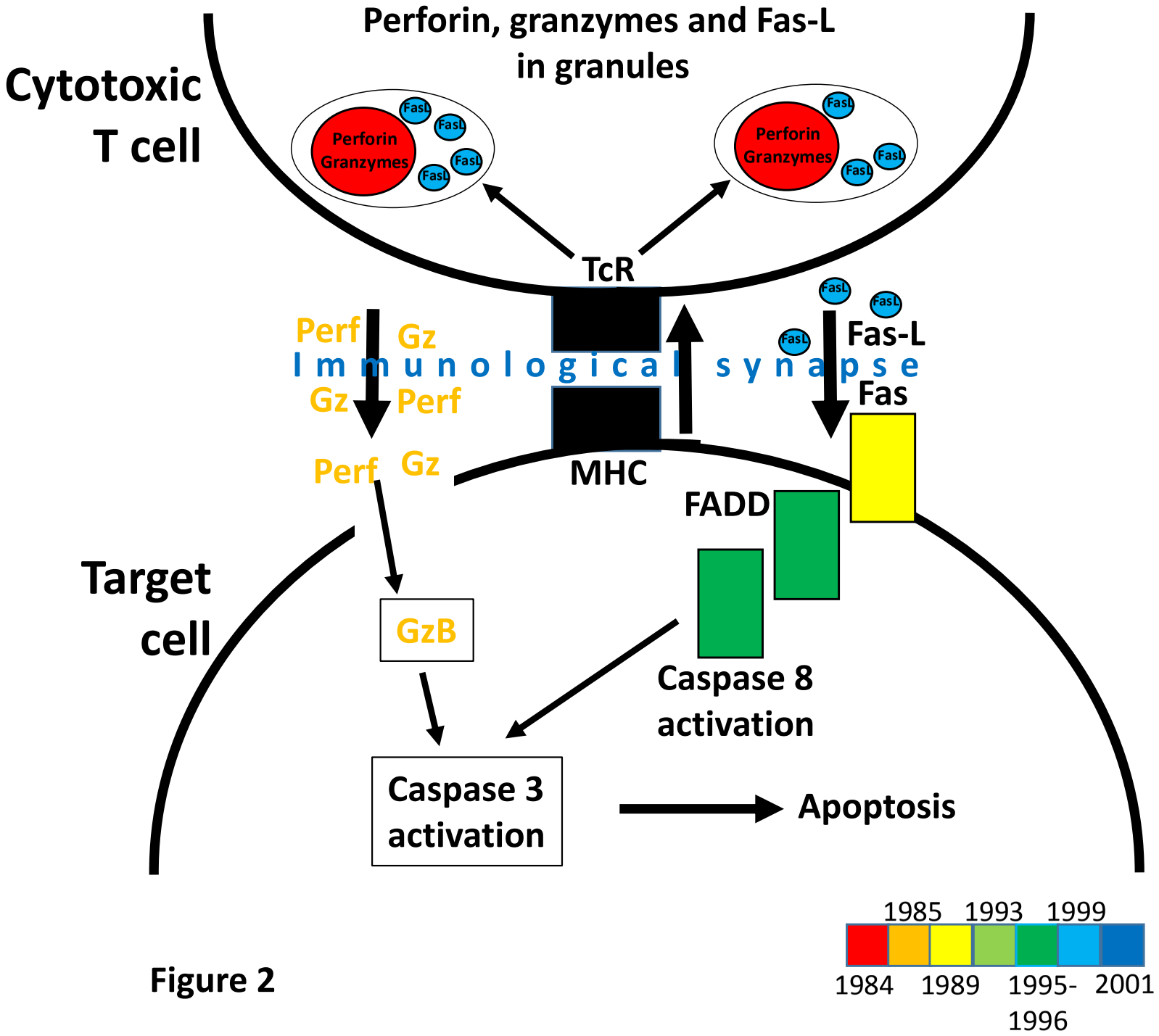
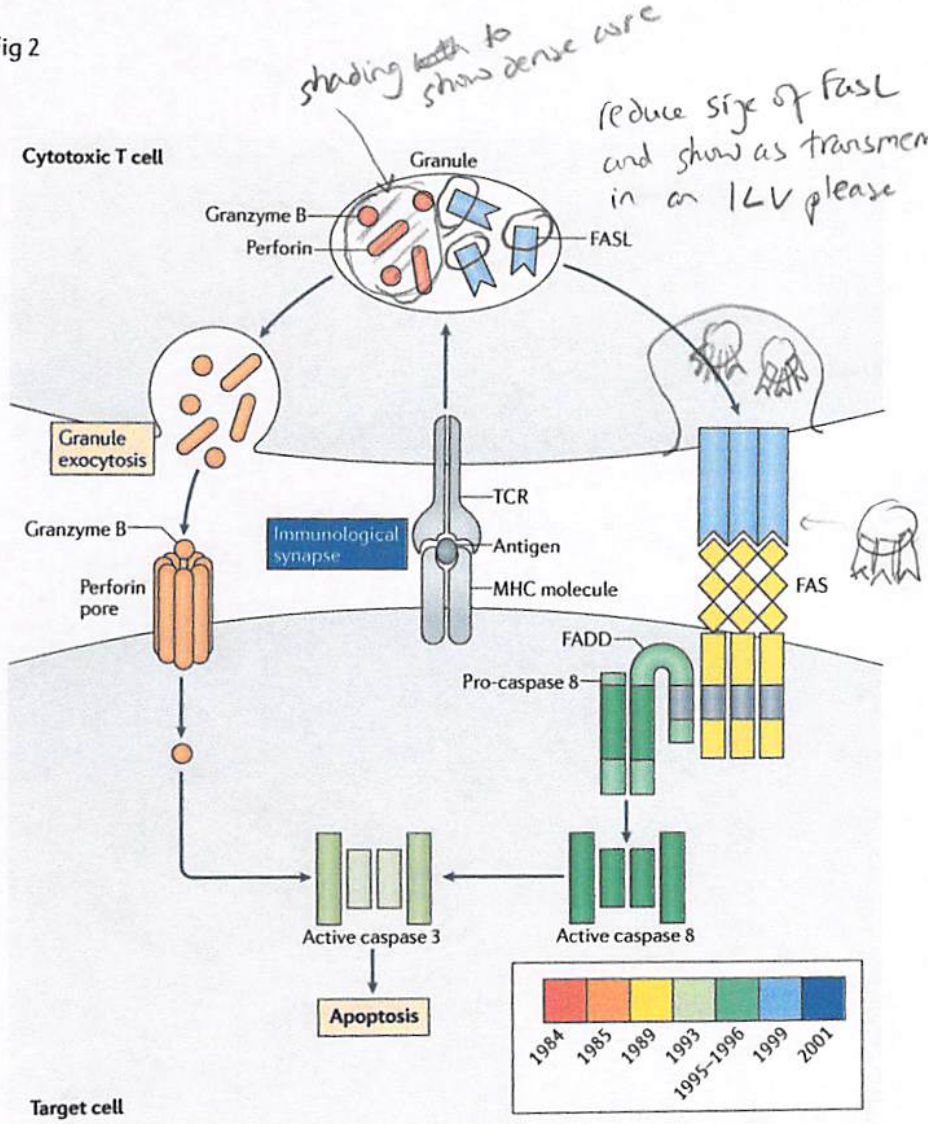

Perforin, granzymes and Fas-L
in granules
Cytotoxic
T cell
Perforin
Granzymes
FasL
TcR
Perf
Gz
Immunological synapse
Gz
Perf
Perf
Gz
Fas-L
Fas
MHC
FADD
Target
cell
GzB
Caspase 8
activation
Caspase 3
activation
Apoptosis
1985
1993
1999
1984
1989
1995-
1996
2001

Figure 2

Fig 2



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