Resistance of tumors to CTL mediated apoptosis

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Abstract: Immunotherapy is a promising strategy in the fight against cancer, based on the mechanism of action of T cells. However tumors have developed several ways to evade this strategy. This is done by interfering with the intrinsic or extrinsic apoptotic cascade, used by T cells to kill tumor cells. This can be done by interfering with molecules involved in the apoptotic cascade or by interfering with regulators of the apoptotic cascade or, by interfering with regulators of regulators of the apoptotic cascade. This interference leads to a reduced sensitivity to CTL mediated lysis or even to a complete resistance against CTL mediated lysis.

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Introduction

The occurrence of tumors and malignancies is due to an imbalance in the life and death cycle of cells [1,2]. An attractive therapeutic strategy against cancers is immunotherapy, which is based on the mechanism of action of T cells. This strategy holds a great promise, due to its tumor specificity, and potentially long term efficacy [3]. However, tumor cells have developed several mechanisms to escape immunotherapy, amongst which immune suppression and apoptosis resistance to immunotherapy [3].The scope of this review is to give an overview of the strategies, which tumor cells use to escape immune mediated apoptosis, specifically the cytotoxic T lymphocyte (CTL) mediated lysis. This overview will be given by first showing how CTL mediated apoptosis should work, after which the mechanism tumors use to evade CTL mediated lysis will be given.

Induction of apoptosis

In cytotoxic T cells, the immune system has effector cells, which can induce apoptosis through two pathways, the death receptor pathway and the exocytosis pathway. After the induction of apoptosis there are two routes leading up to the execution of apoptosis, the intrinsic and the extrinsic pathway [1-7].

Induction of apoptosis through the death receptors

Death receptors are members of the tumor necrosis factor receptor family, which contain a death domain. The known members of the death receptor family are fas (CD95), Death receptor 3,4,5 (DR3, DR4, DR 5) and TNFR1. The death receptors are activated by their natural ligands, like tumor necrosis factor-related apoptosis inducing ligand (TRAIL) for DR4 and DR5 and CD95L/FasL for CD95.These ligands activate the extrinsic cellular death pathway and through the activation of t-BID it is possible that the intrinsic pathway is activated, like the induction of apoptosis through exocytosis (figure 1,2) [1-7].

Induction of apoptosis through exocytosis

Granule exocytosis is another pathway through which CTLs can induce apoptosis. These granules are cytotoxic secretory liposomes containing different granzymes (granzyme B is the only one mentioned in this report) and perforin. These granzymes can induce apoptosis via direct caspase activation and the intrinsic pathway [8,9]. When a CTL recognises an antigen via the antigen receptor, the CTL and its target cell form a well organised immunological synaps [8,9]. After binding to the target cell and the formation of the synaps, the granules are released from the CTL. The perforin creates pores in the membrane of the target cell, through which granzyme B can enter the cell and initiate various routes induce apoptosis. The first route is through the caspases or the extrinsic pathway. Granzyme can also induce apoptosis by the mitochondrial/intrinsic pathway through the activation of BID (see figure 1).

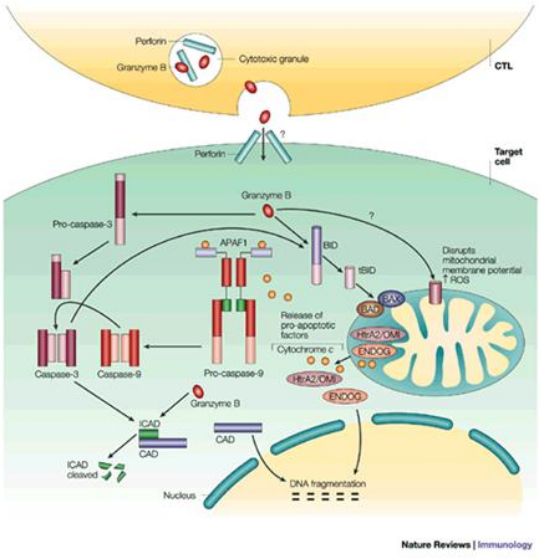

Figure 1: granzyme mediated pathways of apoptosis [8]

Extrinsic pathway of apoptosis execution

When ligands bind to the death receptor, the death of the receptor domain attracts Fas associated death domain protein (FADD). FADD recruits inactive proforms of members of the caspase family. Initiator pro-caspase- 8 and pro- caspase-10 are the caspases attracted to the death-inducing signalling complex (DISC) were self-cleavage occurs. The activated caspases cleave and activate downstream effector/executioner caspases, mainly caspase-3, caspase-6 and caspase-7, which cleave and activate each other (figure 2) [2,4,6,7]. At a certain point the caspases start to cleave cellular substrates, essential for the functioning of the cell, death substrates, amongst others nuclear lamins. These substrates lead to morphological changes, characteristic for cell death, after which DNA fragmentation and apoptosis occurs [2,4,5,6,7].

Intrinsic pathway of apoptosis execution

The intrinsic pathway resembles the extrinsic pathway closely, since several components are the same in both pathways, like the executioners involved.

The intrinsic pathway is activated as a consequence of DNA damage signals, by cleavage of caspases-8 and 10, which can be activated through death receptor activation or as consequence of granzyme B entering the cell cytosol [2,4,5,6,7,8,9].

The DNA damage signals are often initiated by the p53 protein, which is a tumor suppressor gene. These DNA damage signals or granzyme B in the cytosol cause the activation of BID leading through the activation of caspase-8 to the activation of BAX and BAK, which are pro-apoptotic proteins of the Bcl2 protein family (figure 2). These proteins activate the mitochondrion, which upon activation releases cytochrome c, and via apotosome, activation finally leads to caspase- 9 cleavage (figure 2). Caspase- 9 can activate the executioner caspases and induce apoptosis [2,4,5,6,7].

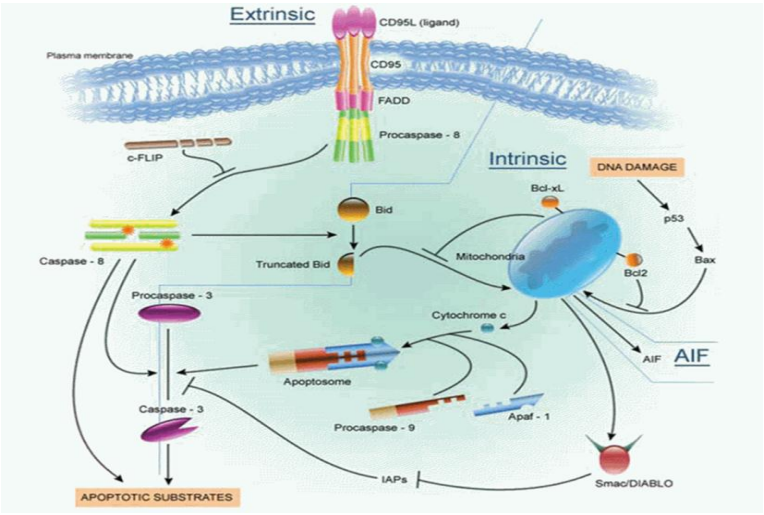

Figure 2: intrinsic and extrinsic pathways of apoptosis [7].

Part two: mechanisms of evasion

Tumors developed several mechanisms through which they can evade CTL mediated lysis. This can be by down-regulating or up-regulating direct regulators of apoptosis, or by influencing regulators of the regulators of apoptosis. Another option is that the tumors have different levels of the proteins that are involved in the apoptotic cascade thus interfering with the apoptotic cascade T cells would induce. One way to do so is by down-regulating proapoptotic proteins of the apoptotic cascade or up-regulating antiapoptotic proteins of the apoptotic cascade. From each of the previously mentioned mechanisms an overview will be given of the potential molecules involved, starting with the members of the apoptotic cascade.

This overview will be given with the use of articles that tested CTLs and tumors or, tumor cells and effector mechanisms of the CTLs, like fas and TRAIL or perforins and granzymes, to see the influence of different expressions of molecules on CTL mediated apoptosis.

Fas, TRAIL and granzymes are important effectors used by CTLs to induce apoptosis in tumor cells, that was the reason to also describe these molecules tested when tested against tumor cells.

Literature research was conducted with amongst others the following terms: antiapoptotic effect, tumor cells and CTLs, or antiapoptotic effect, death receptors and tumor cell, or for granzymes, antiapoptotic effect and CTLs. After finding an indication that a certain molecule influenced apoptosis resistance in tumor cells, further literature research was conducted to that specific molecule.

Members of the apoptotic cascade:

BID

BID is a member of the Bcl-2 family and is a pro-apoptotic gene that is activated by granzyme B or caspase-8, and activates BAX and BAK, which play an essential role in the mitochondrial pathway. The pro-apoptotic proteins from the bcl-2 family are responsible for the permeabilisation of the membrane of the mitochondria and thus the release of cytochrome c from the mitochondria.
BID is a molecule that enhances the death receptor signals by connecting the death receptor pathway to the mitochondrial pathway, which is in some cases required to induce apoptosis [10,11]. BID is essential for myeloid homeostasis and tumor suppression [10]. Upon death receptor activation, the levels of BID increased in TRAIL sensitive cells compared to no increase in TRAIL resistant cells, indicating that down regulation of BID can prevent apoptosis by causing TRAIL resistance [11].
BID was down-regulated in chronic myeloid leukaemia cells which were very malignant, in a similar group, the patients with chronic myeloid leukaemia were BID deficient and resistant to death receptor-induced apoptosis [12].
It has been proven that maintaining normal levels of BID is essential for Death receptor mediated apoptosis of tumor cells, since BID deficiency leads to resistance to death receptor-induced apoptosis. This might indicate that BID is important for CTL mediated lysis, since the death receptor pathway is often used by CTLs to induce apoptosis.

BAX/BAK

BAX and BAK are pro-apoptotic proteins and members of the Bcl2 family, that plays a role in the intrinsic apoptotic pathway (figure 1) [4,13]. BAX and BAK are frequently mutated in tumor cells, which leads to resistance to death receptor induced apoptosis.
In BAX deficient mice it was observed that BAX and BAK are necessary for death receptor induced apoptosis in different cancer cell [13]. Researched showed that colon carcinoma cells depend on BAX for apoptosis, in this malignancy BAX is often down regulated or absent reducing, the survival time [13]. $BAX^{-/-}$ cells were resistant to TRAIL induced apoptosis, thus besides an important role in the mitochondrial pathway another role for BAX is found in the death receptor pathway.
In a study with $BAX^{+/-}$ cells, the cells for TRAIL mediated apoptosis were dependent on the mitochondrial pathway via BAX, in this study apoptosis was inhibited in $BAX^{+/-}$ cells after exposing the cells to TRAIL [14]. It was observed that caspase-8 was still activated but the release of cytochrome c and procaspase-9 were completely inhibited in these cell lines. As a consequence of the inhibition of caspase-9, caspase-3 was also functionally inhibited and the cells were resistant to apoptosis [14].
BAX was shown to be essential for the apoptotic cascade and down-regulation of this molecule leads to apoptosis resistance in tumors due to the evasion of both the death receptor pathway and the mitochondrial pathway.

Bcl-2/Bcl-X

Bcl-2 and Bcl-X are members of the Bcl-2 family, like BID and BAX/BAK, however Bcl-2and Bcl-X are anti-apoptotic proteins, which are often up-regulated in tumors. Both molecules are frequently overexpressed in the more aggressive cancers types [15].

In a breast cancer cell line both Bcl-2 and Bcl-X were identified as in inhibitors of the TNF induced death pathway, when tested against Fas, this pathway of apoptosis was also completely inhibited, while the cells were sensitive to cytotoxicity induced by Fas and TNF when Bcl-X or Bcl-2 were not overexpressed [15].

When Gossypol was used as an inhibitor (a small molecule with moderate affinity for the antiapoptotic proteins of the Bcl-2 family) it was observed that it resulted in tumor regression [16].

When another inhibitor of the Bcl-2 anti-apoptotic family was added to prostate cancer cells there was a significant increase in caspase-3 and caspase-9, a significant decrease in Bcl-X and Bcl-2 and a dose dependent increase in the release of cytochrome c from the mitochondrion [17]. Cytochrome c is needed to activate the executioner caspases in the mitochondrial pathway. Bcl-2 and Bcl-X bind to the mitochondrial membrane and prevent the release of cytochrome c form the mitochondrion, inhibiting the on-going of the mitochondrial pathway [1].

The data provided above indicates that Bcl-2 and Bcl-X when overexpressed in tumor cells can interfere with the intrinsic apoptotic pathway and the death receptor pathway, potentially meaning that there could be interference with CTL mediated lysis.

Direct regulators of the apoptotic cascade:

c-FLIP

One of the molecules potentially involved in the immune evasion of tumors is FLICE inhibitory protein isoform c (c-FLIP).

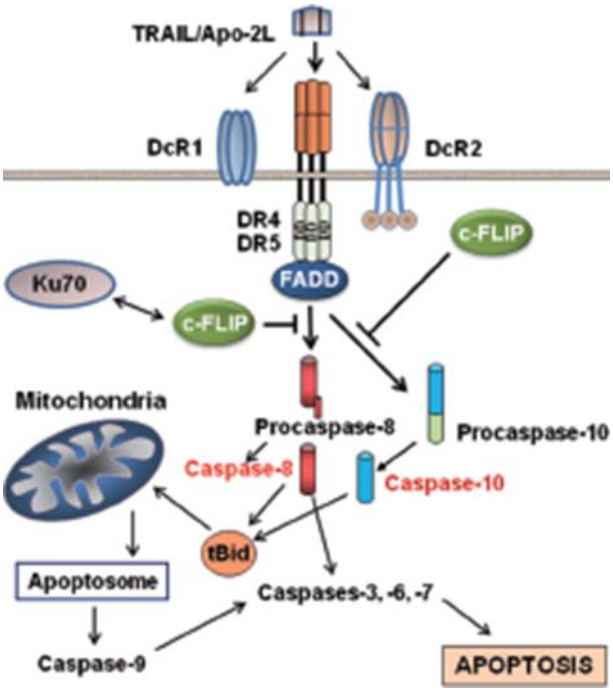

Figure 3: mechanism of c-FLIP [18]

C-FLIP is an inhibitory molecule in the apoptotic cascade, inhibiting DR4 and DR5 induced apoptosis (figure 3) [18-22].
C-FLIP is up-regulated in several cancer types, like lung cancer [18-22].
When silencing c-FLIP$_L$ TRAIL mediated apoptosis was significantly increased compared to cells expressing c-FLIP$_L$ [20].

C-FLIP amounts in a cell were directly correlated with resistance to death receptor induced apoptosis, c-FLIP$_s$ caused resistance to CD95 induced apoptosis and c-FLIP$_L$ caused a resistance to TRAIL and TNFα induced apoptosis [22]. In cells overexpressing c-FLIP$_s$ the formation of the active subunits of caspase-8 was completely inhibited at the DISC, preventing the activation of executioner caspases [22].
These results indicate that c-FLIP is a protein that can prevent death receptor induced apoptosis, a pathway frequently used by CTLs to induce apoptosis. This could indicate that c-FLIP expression might be protective against CTL mediated lysis, by preventing the formation of active caspase-8 and inhibiting death receptor induced apoptosis [22].

Mcl-1

Mcl-1 is an anti-apoptotic protein that is frequently over-expressed in several types of cancer, like leukaemia, prostate cancer and lung cancer [23].
Mcl-1 siRNA can sensitise cancer cells to TRAIL mediated apoptosis. In cholangiocarcinoma cells silencing Mcl-1 caused a significant increase in TRAIL mediated apoptosis [24].
In ovarian cancer cells, Mcl-1 was significantly up-regulated in the ascites; this inhibited the TRAIL mediated apoptosis of these cancer cells. When Mcl-1 was knocked down with siRNA, TRAIL mediated apoptosis significantly increased in these cells [25].
In lung cancer cell lines, Ouabain (an inhibitor of Mcl-1) caused a significant increase in TRAIL mediated apoptosis. After further research, the increment in apoptosis was caused by a significant decrease in Mcl-1, indicating that suppression of Mcl-1 is important for TRAIL mediated apoptosis in tumor cells [26].
The results above indicate that Mcl-1 is an important player in the resistance of cancer against death receptor mediated apoptosis, a strategy often used by CTL to induce apoptosis in cancer; this might indicate that mcl-1 plays a role in the evasion of tumor cells against CTL mediated lysis.

PI-9

Serine protease inhibitor serpin (PI-9) is a member of the intracellular ovalbumin-family. PI-9 is expressed in various cancer cell types like lung cancer and prostate cancer [27,28]. In these cancer cells it has a protective role against granule mediated apoptosis. PI- is an inhibitor of granzyme B, by binding to granzyme B stable complex id formed in which granzyme B is inactivated. An increment in PI-9 caused a decrease in activated caspase-3 this relation was dose-dependent, proving that PI-9 interferes with the apoptotic pathways [27].
PI-9 was detected, at both mRNA and protein level in the highly invasive cancer cell lines PC3 and DU145, whereas none was detected in control cell lines. In the PC3 cell lines granzyme B did not cleave its substrate and formed a stable complex with PI-9, which proves that at a cellular level PI-9 inhibits granzyme B induced apoptosis [28].
In a study with the murine homologue of PI-9, SPI-6 in carcinoma and normal cell lines, SPI-6 was expressed in several cancer cell lines, in which it formed a stable complex with granzyme B, inhibiting granzyme B mediated apoptosis [29].
The combined results mentioned above indicate that PI-9 is capable of inhibiting granzyme mediated apoptosis, a tool often used by CTLs to induce apoptosis in tumor cells, indicating that PI-9 might be important in the resistance against CTL mediated lysis.

p53

P53 is a tumor suppressor gene, which is a stress-activated transcription factor. It can induce cell death as a response to stress signals. P53 plays a role in both the extrinsic and the intrinsic pathway and can activate both the extrinsic apoptotic pathway and proteins in the intrinsic pathway. P53 is frequently down-regulated or mutated in tumors. The mutations often lead to a loss in functionality [30].
P53 plays a role in the granzyme mediated pathway, since a decrease of p53 activity resulted in a decrease in granzyme B mediated apoptosis [30].
In breast cancer cell lines a mutation in p53 caused a significant reduction of TNF induced apoptosis, which indicated that mutations in p53 made these cells less susceptible to death receptor induced apoptosis [31-32].
The results from these studies indicate that p53 is an important molecule and mutations in p53 can

cause resistance to both granzyme mediated apoptosis and death receptor mediated apoptosis, indicating that mutations in p53 might be important in resistance against CTL mediated lysis

NANOG

NANOG is a transcription factor, a protein which is normally expressed in stem cells and induces self-renewal. It is over-expressed in various tumor cells, like breast, prostate and kidney cancer, and in head and neck cancer, the expression of NANOG has been related to less favourable outcomes [34].
A study in non-small cell lung carcinoma confirmed the importance of NANOG in CTL mediated apoptosis. In these cells NANOG was increased and NANOG depletion caused an increase in apoptotic and non-replication proteins. When NANOG was knocked down in this cell line it was observed that the tumour cells were more sensitive to CTL mediated lysis than controls [34].
From the study above it can be seen that NANOG can play a role in resistance against CTL mediated lysis.
The direct effect of NANOG on CTL sensitivity is not straight forward and it is described to be involved in the regulation of several regulators of regulators of the apoptotic cascade, such as STAT3.

miR-210

A strong interest in miRNAs recently evolved, after it was shown that they played a substantial part in tumor progression and immune escape of tumors. One of those miRNAs is miR-210 [35].
miR-210 was significantly up-regulated in several tumour cell lines and played an important role in angiogenesis, tumorogenesis and cell survival.
miR-210 is up-regulated in non-small cell lung carcinoma and melanoma [35]. The tumour cells with high miR-210 levels were not susceptible to CTL mediated lysis. When miR-210 was inhibited the susceptibility to CTL mediated lysis was restored.
This proves that miR-210 is capable of inhibiting apoptosis when up-regulated in tumor cells.

Regulators of Regulators of the apoptotic cascade

STAT3

Signal transducer and activator of transcription 3 (STAT3), is an important molecule in tumor-induced immunosuppression and apoptotic escape. STAT3 has proven to interfere with CTL mediated lysis at many levels [36-39].

STAT3 expression in tumors can be regulated by many factors leading to the phosphorylation and activation of STAT3. STAT3 is up-regulated in several cancer types like, glioblastoma multiforma, head and squamous cell carcinoma, leukaemia, multiple myeloma and lymphomas [40]. In glioblastoma multiforme the levels of STAT3 were significantly increased compared to normal astrocytes and it was constitutively activated [40]. When STAT3 was inhibited in these cells it was observed that Bcl-X, Bcl-2 and Mcl-1 were also reduced [40].

In pancreatic cancer cells STAT3 was knocked down, increasing TRAIL mediated apoptosis, through the down-regulation of Mcl-1, Bcl-X and Bcl-2 [41].

STAT3 down-regulation can lead to an increase in TRAIL mediated apoptosis, indicating that STAT3 might play an important role in evasion of death receptor mediated apoptosis and perhaps CTL mediated lysis.

Besides the role mentioned above STAT3 has many other roles in the immune escape of tumors but these are outside the scope of this report.

FBW7

F-box and WD repeat domain- containing 7(FBW7) is a tumour suppression protein which regulates several oncogenic proteins, amongst others Mcl-1 [42,43]. FBW7 is inactivated or down-regulated in various tumors, like leukaemia, breast cancer and colon cancer [44].

It is a protein that is involved in many processes in cancer, but for the scope of this review only its role in the evasion of CTL mediated lysis and related cell will be described. When FBW7 is depleted, Mcl-1 concentrations are increased significantly, as described previously Mcl-1 is an antiapoptotic protein that can be important in the evasion of CTL mediated lysis by tumor cells. One way in which Mcl-1 can be up-regulated is by the down-regulation of FBW7 [43,44]. This has been observed in several cancers [43,44]. The role of FBW7 in the up-regulation of Mcl-1 has also been researched; it has been uncovered that FBW7 tags Mcl-1 for degradation thus preventing an increased concentration of Mcl-1, when FBW7 was down-regulated tumourigenesis was increased [44]. Another study revealed the same results for Mcl-1 [43].The results mentioned above indicate that FBW7 could be tumor a suppressor protein that might by regulating an antiapoptotic protein that is important in the inhibition of the death receptor pathway, indicating that it is possible that interference with CTL mediated lysis occurs.

Discussion/conclusion

The last couple of years interest in immunotherapy in cancer has developed rapidly, as a promising therapeutic method to defeat cancer. Great advances were made in these years in the field of tumor immunology. However putting the research into practice revealed serious difficulties, tumor cells had harnessed themselves against the human immune system failing to respond to immunotherapy.
The challenges for immunologists, oncologists, and others lye in the mechanisms by which tumors evade the human immune system. One of the mechanisms by which tumors evade the immune system is by being resistant to CTL mediated lysis. The cause of CTL mediated lysis resistance was for the scope of this thesis divided in three classes: members of the apoptotic cascade, which are up or down-regulated leading to resistance to apoptosis; direct regulators of apoptosis, these molecules interfere directly with the apoptotic cascade; and lastly the regulators of the regulators of apoptosis, which regulate the direct regulators of apoptosis.
The last group is the most complex one and also the most difficult to use in potential treatment options. Often these molecule have multiple effects, meaning that modulations of these molecules can have unintended side effects.
The group of direct regulators of apoptosis has the most potential to use as targeted treatment in cancer.
One of the molecules most researched is c-FLIP; this molecule is strongly up-regulated in tumor cells, inhibiting the death receptor pathway, when c-FLIP was silenced apoptosis was significantly increased in cell lines. C-FLIP has been proposed as a therapeutic target, however due to the resemblance of c-FLIP with caspase-8 it is difficult to target c-FLIP with a small molecule. It was tried to target c-FLIP with chemotherapy, this interferes with the transcription of c-FLIP, and this made these cells susceptible to death receptor induced apoptosis [45]. This indicates that targeting c-FLIP with chemotherapy in combination with immunotherapy could induce CTL mediated apoptosis.
Against Bcl-2 one antisense and three small molecules are in clinical trials, the suggestion is that it is needed to inhibit multiple antiapoptotic molecules of the Bcl-2 family to prevent apoptosis inhibition by these proteins, however these molecules are more likely to have severe side effects. This also reveals the problem of cancer immunology; tumors use various mechanisms to evade the human immune system and immunotherapy leading to a very complex situation [46].
It is important to unravel the mechanism tumors use to evade the immune system and base immunotherapy at least for a part on these mechanisms, since only then immunotherapy will have the future. In this review several mechanisms can be found indicating that it is important to research the tumor cells for the expression of potential targets, which can be used to enhance the strength of the immunotherapy above it is mentioned that there are trials with some of the inhibitors of apoptosis leading to apoptosis, this means that it is very important to unravel the mechanisms used by tumors to evade CTL mediated lysis leading to a better understanding of the interactions between tumor cells and the immune system. This would lead to a better understanding in the mechanism of evasion used by tumors and to potential targets for therapy in combination with immunotherapy, then the hurdles for immunotherapy described above could be overcome.

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