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Apoptosis: a *Janus bifrons* in T-cell immunotherapy

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ABSTRACT

Immunotherapy has revolutionized the treatment of cancer. In particular, immune checkpoint blockade, bispecific antibodies, and adoptive T-cell transfer have yielded unprecedented clinical results in hematological malignancies and solid cancers. While T cell-based immunotherapies have multiple mechanisms of action, their ultimate goal is achieving apoptosis of cancer cells. Unsurprisingly, apoptosis evasion is a key feature of cancer biology. Therefore, enhancing cancer cells' sensitivity to apoptosis represents a key strategy to improve clinical outcomes in cancer immunotherapy. Indeed, cancer cells are characterized by several intrinsic mechanisms to resist apoptosis, in addition to features to promote apoptosis in T cells and evade therapy. However, apoptosis is double-faced: when it occurs in T cells, it represents a critical mechanism of failure for immunotherapies. This review will summarize the recent efforts to enhance T cell-based immunotherapies by increasing apoptosis susceptibility in cancer cells and discuss the role of apoptosis in modulating the survival of cytotoxic T lymphocytes in the tumor microenvironment and potential strategies to overcome this issue.

THE DUAL ROLE OF APOPTOSIS IN CANCER IMMUNOTHERAPY

Cancer immunotherapies exploit the immune system to combat cancers and thus have revolutionized the field of immuno-oncology, leading to unprecedented outcomes in relapsed and refractory patients.[1] Especially, modulation of T cell's anticancer activity through immune checkpoint blockade (ICB) (eg, anti-programmed cell death protein-1 (PD-1)/programmed death ligand-1 or anti-cytotoxic T-lymphocytes-associated protein 4 (CTLA-4) antibodies, online supplemental box 1) showed a significant clinical response in a subset of solid and hematologic malignancies. Bispecific antibodies (online supplemental box 1) represent another strategy triggering cancer recognition by T cells.[2] The anti-CD19/CD3 bispecific T-cell engager blinatumomab was approved in 2014 for B-acute lymphoblastic leukemia (B-ALL) and several anti-CD20/CD3, and anti-BCMA/CD3 antibodies are in advanced clinical development (online supplemental box 1).[3–6] Although checkpoint inhibitors are currently the treatment backbone for several cancer types, many patients eventually develop secondary resistance and progressive disease in the end.[7] Chimeric antigen receptor T-cell (CAR-T) therapy, a form of adoptive cell transfer (ACT),[8] has also demonstrated substantial anticancer efficacy in treating relapsed or refractory B-cell leukemias, lymphomas, and multiple myeloma, which resulted in the approval of multiple CAR-T products by the US Food and Drug Administration (FDA) (online supplemental box 1).[9–14] Nevertheless, approximately 50% of pediatric B-ALL and up to 70% of patients with B-cell lymphoma still do not respond or eventually relapse to the CAR-T therapy.[10 12 13 15] Therefore, improving the potency of T cell-based immunotherapies is critical for improving the clinical outcomes of patients with cancer.

The ultimate goal of anticancer therapy, including T cell-based immunotherapies, is to eliminate cancer cells, mainly by efficiently inducing apoptosis in cancer cells. Apoptosis, a programmed cellular mechanism leading to cell death, is a complex biological process involving a vast array of tightly controlled cellular components.[16] The acquisition of resistance to programmed cellular death (eg, apoptosis) is a key feature of cancer progression.[17] For instance, genetic alteration of the anti-apoptotic regulator (eg, translocation and/or gain of B-cell lymphoma 2 (BCL-2)) has been well characterized as a key biological marker in multiple lymphomas, including follicular B-cell non-Hodgkin's lymphoma, diffuse large B-cell lymphoma, and B-cell chronic lymphocytic leukemia (CLL).[18] High levels of BCL-2 expression protect these fast-growing lymphomas against apoptosis, allowing malignant B cells to survive under various stress factors, such as cytokine deprivation. The critical role of

apoptosis in cancer development has been further identified during the transformation of premalignant cells into malignant cells. While MYC expression in premalignant cells increases sensitivity to apoptosis, a similar expression of MYC in malignant cells provides a strong proliferative advantage without inducing apoptosis. This proliferative advantage of MYC expression can be attributed to the co-expression of anti-apoptotic regulators (ie, BCL-2) in malignant cells, indicating that acquiring resistance to apoptosis by increasing expression of anti-apoptotic regulator (ie, BCL-2) during malignant transformation is an important checkpoint in cancer development.[18 19] Considering the critical role of apoptotic resistance in cancer development, this resistance may also provide a strong protective mechanism against T cell-based immunotherapies. Therefore, it is essential to understand not only the general molecular mechanisms of apoptosis but also the evasion mechanism of cancer cells to enhance the anticancer activity of T cell-based immunotherapies.

Multiple cellular insults and external stimuli, broadly categorized as intrinsic or extrinsic, can promote apoptosis. Intrinsic apoptosis is triggered by DNA damage, excessive reactive oxygen species (ROS), hypoxia, or cellular/metabolic stress.[20] In contrast, extrinsic apoptosis is initiated by the so-called 'death ligands,' such as Fas ligand (FasL or CD95L), TRAIL (TNF-related apoptosis-inducing ligand), and tumor necrosis factors (TNFs).[21] Immune cells, particularly T cells, use both pathways to activate apoptosis in cancer cells (figure 1). On T-cell receptor (TCR) engagement, T cells release cytolytic granules containing granzymes and perforin in the immune synaptic space to initiate the intrinsic apoptotic pathway. Perforins are pore-forming proteins that diffuse across immunological synapses and oligomerize

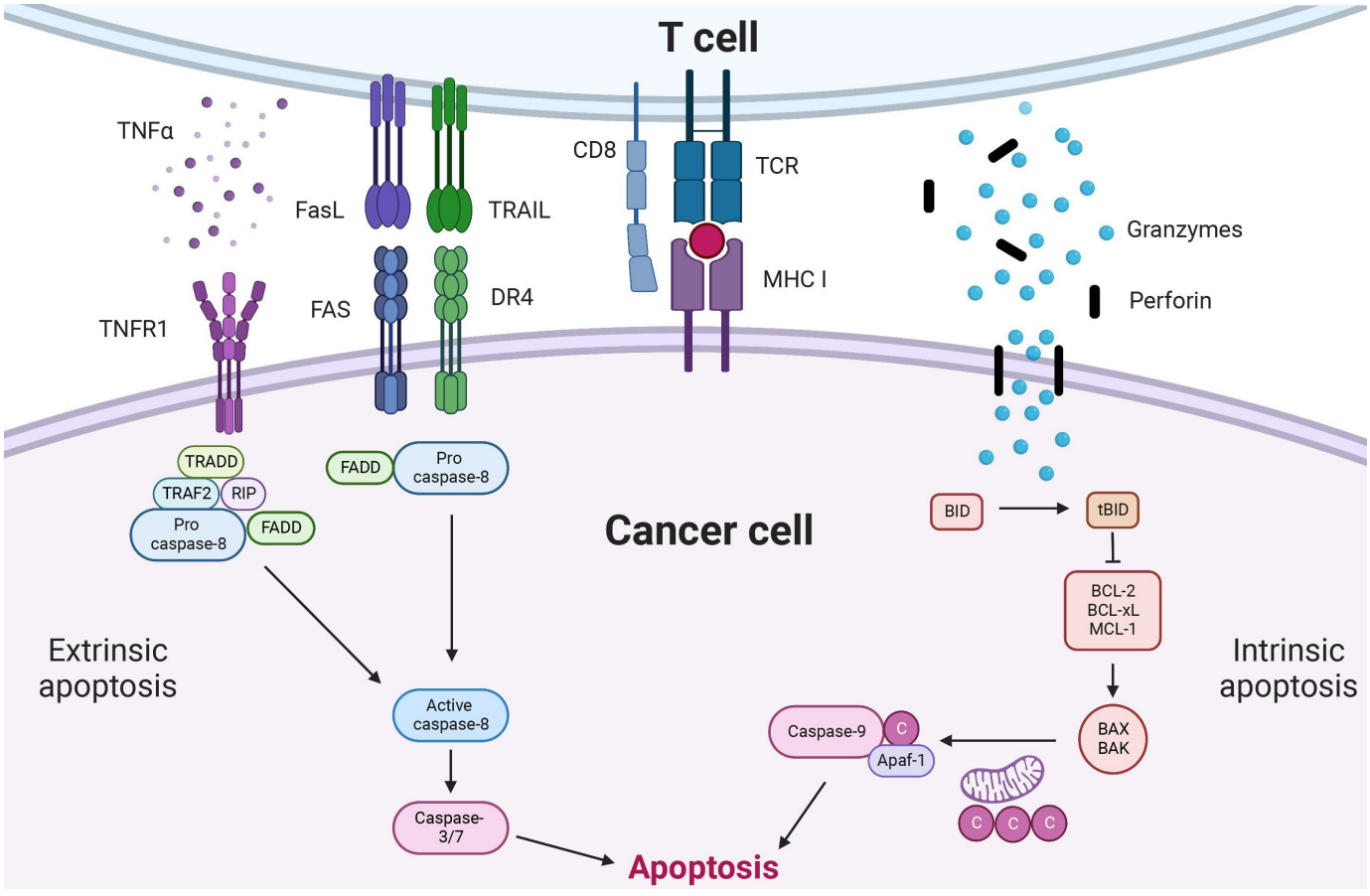

Figure 1 Apoptosis induced by T cells. T cells can induce apoptosis in cancer by both extrinsic and intrinsic pathways. To promote intrinsic apoptosis in cancer, granzymes is first transported into cancer cell via perforin, and granzymes cleave BID to generate truncated BID (tBID). tBID inhibits anti-apoptotic regulators (eg, BCL-2, BCL-xL, MCL-1), leading to the formation of homodimer or heterodimer of BAX and BAK on the membrane of mitochondria. These BAK/BAX dimerization releases cytochrome C and Apaf-1 from mitochondria to the cytoplasm. Together with Caspase-9, cytochrome C and Apaf-1 form apoptosomes that can cause apoptosis in cancer cells. FasL, TRAIL, and TNF-α expressed by T cells engage with their corresponding receptor in cancer cells to initiate extrinsic apoptosis. On engagement, death receptor complexes are formed and activate Caspase-8. Finally, activated Caspase-8 stimulates the activity of Caspase-3/7, resulting in the induction of apoptosis in the cancer cell. C: cytochrome C, BCL-2, B-cell lymphoma 2; FADD, Fas-associated death domains; FasL, Fas ligand; MHC, major histocompatibility complex; TCR, T-cell receptor; TNF, tumor necrosis factor; TRADD, TNF receptor type 1-associated death protein; TRAIL, TNF-related apoptosis-inducing ligand.

to form pores on the target cell membrane, facilitating the entry of granzymes into the target cell.[22] Granzymes generally enter target cells through pores formed by perforin; it has also been described that granzymes can pass the cell membrane of target cells without requiring perforin, via pinocytosis.[23] Four subsets of granzymes (granzyme A, B, K, and M) have been identified in human T cells; particularly granzyme B plays an essential role in promoting T cell-induced apoptosis in target cells.[24] On entry into the target cell, granzyme B cleaves BH3 Interacting Domain Death Agonist (BID), a BCL-2 homology (BH3)-only pro-apoptotic protein that plays an essential role in promoting apoptosis. Cleavage of BID results in the generation of the active form of BID (truncated BID/tBID). Subsequently, tBID activates pro-apoptotic effector proteins, such as BAX and BAK, by interfering with their interaction with BCL-2. This activation of effector proteins leads to the induction of instability of the mitochondrial membrane potential and the release of cytochrome c from the mitochondria to the cytoplasm. Cytochrome c leads to the formation of apoptosome complexes (cytochrome c:APAF-1:Caspase-9), which activate effectors, Caspase-3 and Caspase-7, promoting downstream apoptotic signaling cascades.[25] However, to trigger the extrinsic apoptotic pathway, the engagement of T cell-derived death ligands and their associated receptors in target cells is necessary. When T cells are activated, it leads to an increase in the expression of death ligands, such as FasL, TNF-α, and TRAIL. These death ligands bind to their respective death receptors (eg, FasL-Fas (CD95), TNF-α-TNFRSF1A (TNFR1), and TRAIL-TNFRSF10A (DR4)) on target cells, triggering the formation of death-inducing signaling complexes (DISCs), comprizing adaptor proteins (eg, Fas-associated death domains (FADD) or TNF receptor type 1-associated death protein (TRADD)) and initiator Caspase-8. DISCs eventually initiate downstream apoptotic signaling cascades to induce cellular apoptosis.[25]

Cancers have developed several strategies to evade immune cell-mediated apoptosis. For instance, mutation of TP53, a key tumor suppressor gene that confers resistance to apoptosis is strongly associated with decreased immune function genes (eg, granzymes and perforin) in patients with gastric cancer.[26 27] This observation suggests that aberrant TP53 activity could affect the anticancer immune response. Modulation of anti-apoptotic (eg, BCL-2, CFLAR, and BIRC2) and pro-apoptotic proteins (FAS, FADD, TNFRSF10B (death receptor 5—DR5), BID, and Caspase-8) is another important mechanism used by cancer cells to blunt cancer immunotherapy's anticancer efficacy.[28–33] Maruyama *et al* reported that >40% of patients with metastatic renal cell cancer with no response or progressive disease were positive for immunohistochemical staining of BCL-2, while patients with complete or partial responses were negative for BCL-2 during the treatment course of immune-stimulatory treatments (eg, interferon (IFN)-α, IFN-γ, and interleukin-2).[28] Furthermore, we performed a retrospective analysis of the clinical response of patients with lymphoma treated with anti-CD19 CAR-T therapy and showed that patients with genetic alterations in BCL-2 (ie, gain or translocation of BCL-2) show significantly lower response and overall survival to CAR-T treatments than patients without genetic alterations of BCL-2,[33] implicating that genetic alteration of BCL-2 plays a crucial role in the anticancer efficacy of CAR-T therapy. In addition to the effect of altered intrinsic regulators of apoptosis on cancer immunotherapy, our group also demonstrated that CAR-T cells' anticancer efficacy is significantly reduced when leukemic cells display decreased expression of positive regulators of apoptosis, especially in the death receptor pathway (FasL, TRAIL, and TNF-α).[34] By using unbiased genome-wide CRISPR knock-out (KO) screening, we identified that the deletion of anti-apoptotic regulators (eg, BIRC2, CFLAR, and TRAF2) in the B-ALL cell line NALM-6 led to significant enhancement of the anticancer activity of CAR-T cells, while KO of pro-apoptotic regulators (eg, FADD, Caspase-8, BID, and TNFRSF10B) resulted in a decrease of anticancer activity of CAR-T cells. Further validation with clinical data revealed that the downregulation of pro-apoptotic regulators was significantly associated with a poor clinical response in patients with B-ALL treated with anti-CD19 CAR-T cells. Likewise, Upadhyay *et al* showed that Fas-FasL-mediated cancer killing plays a crucial role in T cell-based immunotherapy, and the expression of Fas in cancer strongly correlates with the clinical outcome of CAR-T therapy.[29] Importantly, these studies suggest that mechanisms conferring resistance to apoptosis in some cancer cells can also drive T-cell dysfunction, leading to poor clinical outcomes of T cell-based immunotherapy.

Given that resistance to apoptosis in cancers could be a critical factor associated with poor clinical outcomes of T-cell mediated immunotherapy by causing dysfunction of T cells, this review will highlight several novel therapeutic strategies designed to augment T cell-mediated cancer apoptosis. Furthermore, it discusses tumor-derived or tumor microenvironment (TME)-derived factors that govern T-cell apoptosis and rational strategies to prevent it.

STRATEGIES TO ENHANCE T-CELL MEDIATED CANCER APOPTOSIS

Despite remarkable clinical outcomes of T cell-based immunotherapies, a substantial number of patients do not benefit from these approaches.[9–15] Considering the importance of cancer apoptosis susceptibility in the cytolytic activity of T-cell therapy, several interesting therapeutic approaches have been investigated to overcome apoptosis resistance in cancer cells (figure 2 and table 1).

First, researchers have tested whether conventional anticancer therapeutics, such as chemotherapy or radiotherapy, can enhance cancer apoptosis during immunotherapy. Both, chemotherapeutic agents (eg, alkylating agents, anthracyclines, vinca alkaloids, and antimetabolites) and radiation (eg, X-ray), potently promote intrinsic

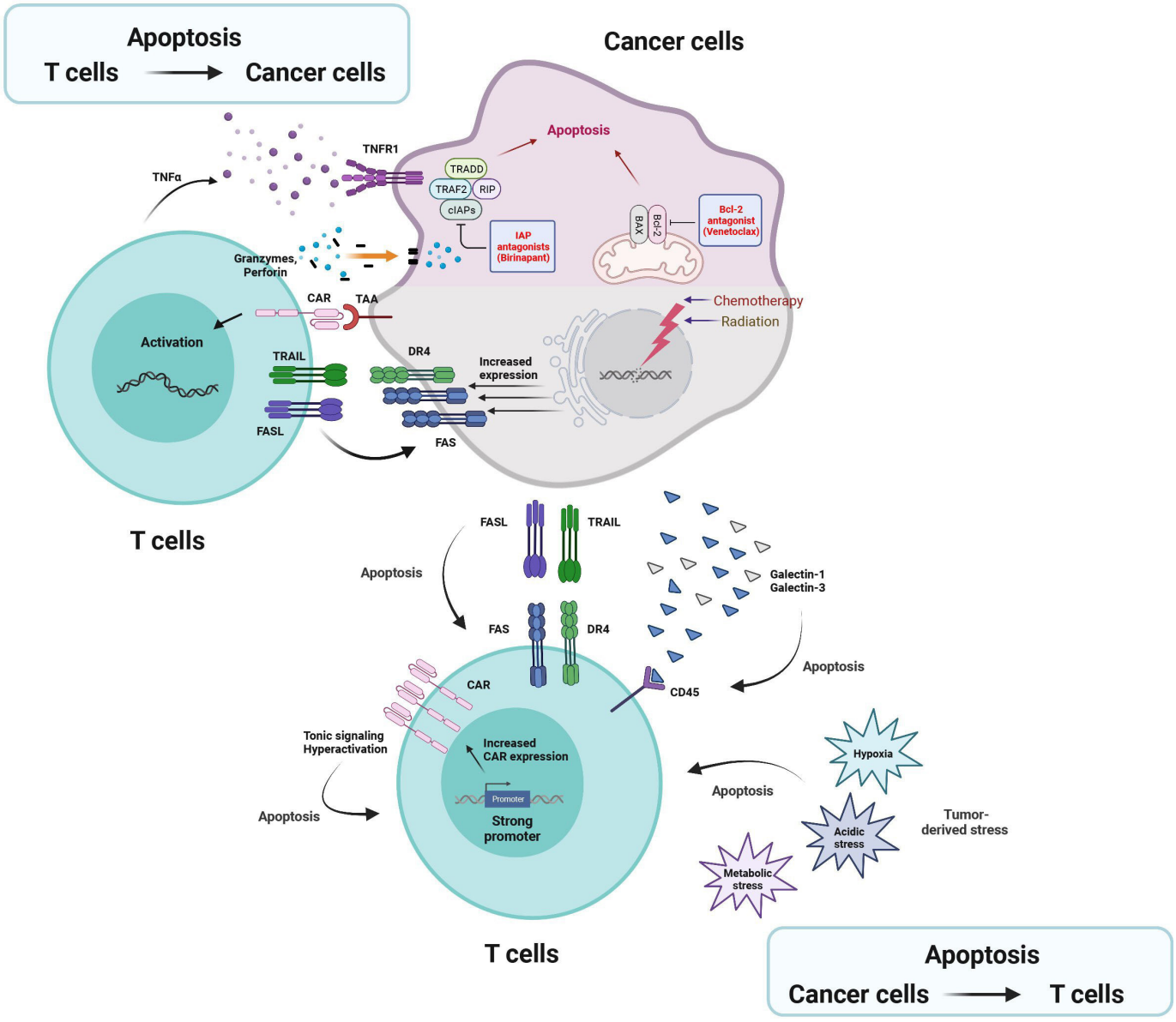

Figure 2 Dual effects of apoptosis in T cell-based immunotherapy. On the cancer cells side, chemotherapy and radiation therapy induces the expression of DR4 and Fas in cancer cells, sensitizing cancer cell to TRAIL-mediated and FasL-mediated apoptosis. In addition, treatment of small molecules that can specifically inhibit anti-apoptotic regulators (eg, IAP and BCL-2) leads to the enhancement of cancer apoptosis mediated by TNF-α or granzymes/perforin. On the T-cell side, multiple factors (eg, FasL, TRAIL, and galectin) and stress (eg, hypoxia, metabolic alteration, and acidification) derived from cancer cell and tumor microenvironment promote apoptosis in T cells. Hyperactivation and tonic signaling of CAR-T cells by increased CAR expression on the surface induce apoptosis in T cell. BCL-2, B-cell lymphoma 2; CAR, chimeric antigen receptor; cIAP, cellular IAP; DR4, death receptor 4; FasL, Fas ligand; IAP, inhibitor of apoptosis proteins; TAA, Tumor associated antigen; TNF, tumor necrosis factor; TRADD, TNF receptor type 1-associated death protein; TRAIL, TNF-related apoptosis-inducing ligand.

apoptosis in cancer cells by inducing DNA damage and/or inhibiting cell cycle.[35 36] Moreover, treatment with selected chemotherapies (eg, etoposide, doxorubicin, and 5-fluorouracil) and radiation can also modulate death receptor-mediated extrinsic apoptosis in cancer cells by affecting the transcriptional activity of death receptors (eg, Fas and TNFRSF10 families).[37–41] On exposure to etoposide, DR5 and Fas expression increased in human lung, colorectal, prostate, bladder, and breast cancer cell lines, leading to improved immune cell (natural killer T cell-mediated killing).[37] Similarly, sublethal doses of doxorubicin upregulate TRAIL receptors on cancer cells (eg, MAR and JOHW colorectal carcinoma cell lines), promoting natural killer and tumor-infiltrating lymphocyte (TIL) cytotoxicity.[39]

Interestingly, the authors also observed that doxorubicin treatment reduced the expression of intracellular FLICE inhibitory protein (c-FLIP), a key anti-apoptotic inhibitor of death receptor-mediated apoptosis. This finding suggests that doxorubicin can sensitize cancer cells to immune cell-mediated extrinsic apoptosis by modulating both pro-apoptotic and anti-apoptotic regulators.

Table 1 Summary of the preclinical combinations of pro-apoptotic drugs and T cell-based immunotherapies

Class	Drug	Target	Combination	Cancer type	Effect	Ref
Chemo therapy	Cisplatin, etoposide	DNA damage	NKT cells	NSCLC cell lines CRC cell lines PC cell lines BC cell lines	Sensitization to TRAIL-mediated and FasL-mediated apoptosis	37
Chemo therapy	Doxorubicin, 5-fluorouracyl	DNA damage	Vγ9Vδ2 T cells	CRC cell lines	Sensitization to TRAIL-mediated apoptosis	38
Chemo therapy	Doxorubicin	DNA damage	NK or T cells	Melanoma and bladder cancer cell lines	Sensitization to TRAIL-mediated apoptosis	39
Radiation	Sublethal irradiation	–	antitumor CTLs and NK cells	CRC cell lines	Sensitization to TRAIL-mediated and FasL-mediated apoptosis	40
Radiation	Sublethal irradiation	–	CEA-specific HLA-A2-restricted CD8(+) CTLs	23 human carcinoma cell lines (12 colons, 7 lungs, and 4 prostate)	Sensitization to FasL-mediated apoptosis	41
SMAC mimetic	Birinapant	Inhibition of XIAP and cIAP1/2	anti-CD19 CAR T cells	B-ALL	Increase of CAR T cell-mediated apoptosis	34
SMAC mimetic	Birinapant	Inhibition of IAPs	anti-HER2 CAR T cells	HER2+patient-derived colorectal tumoroids	Sensitization to TNF-α-mediated apoptosis	56
SMAC mimetic	Birinapant	Inhibition of IAPs	anti-CD19 CAR T cells	B-ALL	Sensitization to TNF-α-mediated apoptosis	32
SMAC mimetic	ASTX660	Inhibition of IAPs	cytotoxic TIL	HNSCC	Enhanced immunogenic cell death	59
BH3 mimetic	ABT737	Bcl-2 family anti-apoptotic proteins	anti-CD19 CAR T cells	Patients with childhood precursor-B ALL	Increase of CAR T cell-mediated apoptosis	65
BH3 mimetic	Venetoclax	Bcl-2 family anti-apoptotic proteins	anti-CD19 CAR T cells	B-ALL and B-lymphoma cell lines	Increase of CAR T cell-mediated apoptosis	67

B-ALL, B-cell acute lymphoblastic leukemia; BC, breast cancer; BCL-2, B-cell lymphoma 2 ; CAR, chimeric antigen receptor; CRC, colorectal cancer; CTL, cytotoxic T cell; FasL, Fas ligand; HER2, human epidermal growth factor receptor-2; HNSCC, head and neck cancer; IAP, inhibitor of apoptosis proteins; NK, natural killer; NKT, natural killer T; NSCLC, non-small cell lung cancer; PC, prostate cancer; SMAC, second mitochondria-derived activator of caspase; TIL, tumor-infiltrating lymphocyte; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand .

Another evidence of chemotherapy-induced immune killing reported that 5-fluorouracil treatment upregulates the expression of DR5 in colon cancer-initiating cells in vitro, leading to enhancement of T cell-mediated cytotoxicity.[38] In addition to chemotherapeutic agents, irradiation, which causes DNA damage and intracellular stress, can also lead to the induction of death receptor-mediated cancer cell apoptosis. For instance, a sublethal dose of irradiation can upregulate the expression of Fas and DR5 in colorectal carcinoma cell lines, making them susceptible to TRAIL-induced and Fas-induced apoptosis.[40] Further investigation identified that a sublethal dose of irradiation can increase Fas expression in over 40% of the cancer cell lines, including colon, lung, and prostate cancers.[41] This suggests that radiation may be an effective strategy in combination with T cell-based immunotherapy to enhance the sensitivity of extrinsic apoptosis in cancer cells. In addition to the modulation of cytolytic activity of endogenous T cells in radiation therapy and chemotherapy, the use of recombinant anti-Fas and anti-DR4/5 agonists along with radiation and chemotherapeutic agents has been investigated. Treatment with recombinant TRAIL combined with bortezomib, vorinostat (SAHA), and valproic acid significantly induced cancer cell apoptosis by sensitizing cancer cells to extrinsic apoptosis signal.[42–45]

Finally, with ample preclinical evidence that chemotherapy and radiation therapy can increase the sensitivity of cancer cells to T cell-mediated apoptosis, various clinical trials exploiting the combination of immunotherapy and chemo/radiation have been registered and conducted (see online supplemental table 1). First, the combination of pembrolizumab (online supplemental box 1) and chemotherapy (ie, carboplatin and either paclitaxel or nanoparticle albumin-bound-paclitaxel) significantly improved overall survival and progression-free survival in patients with metastatic squamous non-small cell lung cancer (NSCLC) as compared with chemotherapy only treated patients.[46] Another clinical investigation using an immunotherapeutic combination (nivolumab and ipilimumab, online supplemental box 1) with chemotherapy (carboplatin, paclitaxel, pemetrexed, and cisplatin) showed similar results.[47] These two independent clinical trials eventually led to FDA approval of chemotherapy in combination with checkpoint blockade for first-line metastatic squamous NSCLC treatment. Despite the substantial

synergy between immune checkpoint inhibitors and chemo/radiation therapy, one caveat remains: apoptosis of T cells can also be increased by chemotherapy and radiation due to the lack of ability of chemotherapy and radiation to distinguish target cells (ie, cancer cells) and effector cells (ie, T cells). The resistance mechanisms of T cells to chemotherapy-induced and radiation-induced apoptosis remain largely unknown. Several studies have highlighted that memory T cells can escape apoptosis triggered by chemotherapy and irradiation, implicating the potential role of memory T cells in synergy strategies.[48 49] Considering the resistance of memory T cells to apoptosis, one possible explanation is that a high level of BCL-2[50] and low level of Bcl-2-like 11 (BIM)[51] expression in memory T cells may increase the threshold of apoptotic sensitivity, allowing them to evade chemotherapy-induced and radiation-induced apoptosis. However, further investigations are required to fully understand survival mechanisms of T cells during chemotherapy and radiation.

While chemotherapy and/or radiation increase the sensitivity of cancer cells to apoptosis, direct inhibition of apoptotic regulators has also been observed in combination with T cell-based immunotherapy. One example is the inhibitor of apoptosis proteins (IAPs) that are overexpressed in many cancers[52] and include several members, such as cellular IAP1 (cIAP1), cIAP2, X-linked IAP (xIAP), neuronal apoptosis inhibitory protein (NAIP), livin, and survivin.[53] cIAP1 plays a critical role in inhibiting TNF-α-mediated apoptosis by preventing the formation of the apoptotic complex (FADD/RIPK1/Caspase-8). Moreover, xIAP inhibits apoptosis by blocking Caspase-3 and Caspase-7 by directly binding to them.[54] Given the importance of IAPs in inhibiting apoptosis in cancer, multiple agents have been investigated to promote IAP degradation, thereby sensitizing cancer cells to apoptosis. Second mitochondria-derived activator of caspase (SMAC) mimetics are small synthetic molecules whose structural and functional features are similar to SMAC, which are endogenous antagonists of IAPs. Several SMAC mimetics (eg, birinapant, LCL-161, ASTX660, Debio1143, BV-6, GDC-0152, CUCD-427, HGS1029, and AT-406) have been developed (online supplemental box 2).[55] Particularly, birinapant has been extensively evaluated for its anticancer properties, including in combination with T cell-based immunotherapies such as immune checkpoint inhibitors, such as anti-PD-1 and anti-CTLA-4 antibodies, driving significantly enhanced TNF-α-mediated cancer cell apoptosis and increase in survival in a murine glioblastoma (GBM) model.[32 34 56 57] Birinapant also improved the anticancer efficacy of CAR-T therapy in murine models. Treatment with birinapant enhanced anti-human epidermal growth factor receptor-2 CAR-T cells-mediated cancer killing by sensitizing cancer cells to TNF-mediated apoptosis.[56] Likewise, Song *et al* also found that treatment of birinapant enhances CAR-T cell-mediated tumor killing in GBM model.[58] Using a CRISPR Cas9 KO library, key mediators of synergy between CAR-T cells and birinapant such as RIPK1, FADD, and TNFRSF10B in cancer cells are identified.[32] Our group also demonstrated that birinapant treatment improved CAR-T cells ability to eliminate B-ALL, which otherwise lacks sensitivity to extrinsic apoptosis[34]; however, these results were obtained in vitro and in vivo validation is required to exclude toxicity on CAR T cells. In addition to birinapant, Ye *et al* studied the combinatorial efficacy of cytotoxic TILs and ASTX660, another antagonist of cIAP1/2 and xIAP, in a preclinical model with head and neck squamous cell carcinoma (HNSCC).[59] The authors found that ASTX660 treatment induced—in the presence of TNF-α—calreticulin (CRT) expression, heat shock proteins 70/90, and high mobility group protein on the surface of human HNSCC cell lines (eg, UMSCC-46 and UMSCC-47). These are key molecular signatures for immunogenic cell death (ICD), suggesting that ASTX660 and TNF-α promoted ICD in HNSCC cell lines. ASTX660-mediated induction of ICD was further confirmed in syngeneic murine cancer models (HNSCC) when combined with radiation. Interestingly, the authors identified that ASTX660 treatment plus TNF-α led to clonal expansion of antigen-specific T-cell clones. This might be due to the enhancement of the antigen-processing machinery in cancer cells, as evidenced by the upregulation of critical components of the antigen-processing machinery (eg, human leukocyte antigen (HLA)-A, HLA-B, HLA-C, ERp57, CRT (intracellular), Transporter associated with antigen processing 1 (TAP1), and TAP2) in human HNSCC cell lines after exposure to ASTX660 and TNF-α.

Members of the BCL-2 family, such as BCL-2, BCL-XL, MCL-1, BAX, and BAK, play a critical role in regulating intrinsic apoptosis by modulating the permeabilization of the mitochondrial membrane.[60] As previously discussed, upregulation of BCL-2 activity via overexpression or translocation is one of the key features of various cancers.[61] Several BCL-2 inhibitors have been developed, including obatoclax, AT101, ABT737, S-055746, S65487, PNT-2258, navitoclax, and venetoclax (online supplemental box 2).[62] In particular, venetoclax, an orally available small-molecule inhibitor with high specificity to BCL-2, has demonstrated substantial anticancer efficacy in treating CLL, other lymphomas, and acute myeloid leukemia, leading to its FDA approval in these settings.[63] Recently, Kohlhapp *et al* reported that venetoclax could enhance the anticancer efficacy of anti-PD-1 antibody (MDX-1106) treatment.[64] Interestingly, the authors found that venetoclax treatment increased tumor infiltrating effector memory T cells, which could also explain the potential role of memory T cells in synergy with pro-apoptotic drugs. The beneficial effects of venetoclax in T cell-based immunotherapy were further identified by our group. Using a combination of venetoclax and CAR-T therapy, we demonstrated a significant improvement in CAR-T cells' anticancer activity against various lymphoma and leukemia xenograft models (eg, OCI-Ly18, MINO, NALM6, KG-1, and MOLM-14).[33] In addition to venetoclax, the combinatory effect of different BCL-2 inhibitors (ie, ABT737) with CAR-T therapy was tested, and it was

found that adding ABT737 to CART19 resulted in an increase in Caspase 3/7 activity in cancer cells, leading to cancer killing enhancement.[65]

While, increasing cancer cell sensitivity to apoptosis using aforementioned pro-apoptotic molecules results in the enhancement of the anticancer response of T cell-based immunotherapies in some models, one potential concern of this approach is the unintended toxicity of these agents on effector immune cells such as T cells, which could be critical for the long-term efficacy of combination immunotherapy. A study showed that SMAC mimetic (ie, LBW242) treatment significantly inhibited virus-specific $CD8^+$ T-cell expansion in vivo by inducing T-cell apoptosis, ultimately leading to the failure of virus replication.[66] Moreover, although Lee *et al* and Kohlhapp *et al* demonstrated that venetoclax augmented the anticancer response of CAR-T therapy and anti-PD-1 treatment, they also found that co-culture of venetoclax with genetically non-modified T cells and CAR-T cells potently reduced their viability.[33 64] These observations strongly suggest that careful design of combination therapies and the sequence of administration are required to avoid T-cell toxicity and ensure long-term therapeutic efficacy. One possible administration strategy to avoid bystander effects on T cells is to pretreat the cancer cells with cytotoxic drugs. Recently we reported that patients with lymphoma receiving venetoclax during bridging therapy prior CAR-T cell infusion achieved significant improvement in clinical response compared with patients treated with no venetoclax-included bridging therapy.[33] In line with our clinical observations, pretreatment of cancer cells with venetoclax enhanced CAR-T cell-mediated anticancer activity in vitro.[67] These preclinical and clinical data strongly suggest that pre-sensitizing cancer cells with anti-apoptotic inhibitors could enhance the anticancer effect of T cell-based immunotherapy while reducing toxicity to T cells.

T-CELL APOPTOSIS LIMITS ANTICANCER IMMUNITY IN THE TME

Apoptosis in cancer therapy could induce both cancer cells to die and result in T-cell death. T-cell apoptosis is an *indirect* result of multiple immunosuppressive mechanisms in cancer genesis. For example, T-cell dysfunction, such as exhaustion, is a physiological state in which T cells lose their effector functions while maintaining viability. Prolonged exhaustion ultimately leads to T cells undergoing cellular apoptosis.[68] Furthermore, the immunosuppressive TME, including immunosuppressive immune cells (eg, T regulatory cells, tumor-associated macrophages, and myeloid-derived suppressor cells)[69 70] and lack of key nutrients (ie, low arginine and changes in available metabolites)[71–73] also have substantial effects on the proliferation and survival of cytotoxic T cells in the TME. Because there are already extensive revisions of the literature on T-cell exhaustion[74–76] and other immunosuppressive factors,[77–79] we focused on the mechanisms of immune evasion that *directly* trigger apoptosis in T-cells.

On activation, T cells enhance the expression of pro-apoptotic proteins (eg, FasL, TRAILs, and TNF), potentially promoting the death of target cells as well as death receptors on their surface (Fas, TRAIL receptors, TNF receptor). This upregulation of death receptors increases the susceptibility of activated T cells to apoptosis.[80 81] This process is called activation-induced cell death (AICD) and plays a vital role in maintaining peripheral immune tolerance and preventing autoimmune disease development.[82] Cancer cells can take advantage of this T-cell liability by using it as a potential immunoevasion strategy. Reports in the late 1990s demonstrated that FasL expression in several malignancies (melanoma, colon, head/neck, liver, and lung) serves as a mechanism of cancer evasion.[83–86] This phenomenon, coupled with evidence that T cells increase the expression of Fas on activation, highlights that cancers can induce apoptosis in T cells via the extrinsic pathway to evade immune surveillance. There has also been evidence of upregulation of TRAIL in a few malignancies (melanoma, liver, breast, and lung), although its correlation with the clinical outcome has been controversial.[87–90] While Bron *et al* found no correlation with prognosis in patients with melanoma,[87] Cross *et al* observed a negative association between TRAIL expression in breast cancers and the clinical outcome of patients with breast cancer.[88] Moreover, heterogeneity exists in the ubiquity of FasL and TRAIL expression across cancers. Another captivating aspect of this mechanism is the observation that cancer can secrete exosomes expressing FasL and independently induce T-cell apoptosis.[91–93] Such observations amplify the potency of FasL-mediated apoptosis of T cells directed by cancer cells, which may cause peripheral T-cell dysfunction.[94]

This cancer-induced, death receptor-mediated T-cell apoptosis has been proven to directly hinder responses to immunotherapy.[95–99] Zhu *et al* used a novel autochthonous melanoma mouse model to demonstrate that FasL-mediated T-cell apoptosis facilitates cancer resistance to anti-CTLA-4 antibody, anti-PD-1 antibody, and ACT.[98] Similar to TCR-mediated activation, CAR-driven T-cell activation also increases the susceptibility of CAR-T cells to apoptosis by upregulating death receptors and associated ligands on their surface. Hyperstimulation of CAR-T cells by incorporating two co-stimulatory domains (CD28 and 4-1BB) also increases Fas and DR5 expression and promoted CAR-T cells apoptosis.[96 97] Lastly, tonic signaling of 4-1BB co-stimulation due to greater anti-CD19 CAR expression driven by a strong promoter, such as retroviral long terminal repeat, increases levels of FasL, leading to apoptosis of CAR-T cells on activation.[99] While introducing multiple co-stimulatory domains into CAR construct was intended to enhance activation,[95] these data present a potential concern of overstimulation suggesting a need of 'modulating' CAR-activation in T cells rather than just boosting it.

Cancer and TME cells also secrete factors that can directly trigger T-cell apoptosis.[100 101] Galectins are a family of proteins produced and secreted by various cells, including cancer and immune cells.[102] Galectins bind to β-galactosides on glycoproteins and glycolipids via a conserved carbohydrate recognition domain, thereby regulating miscellaneous biological events, including apoptosis.[100 101] Many studies have demonstrated that cancer-secreted galectin-3 (Gal-3) can induce T-cell apoptosis in various cancers, including melanoma, lung, and colorectal cancer, on binding to their target TCRs, such as CD7, CD29, CD45, and CD71.[103–108] Mechanistically, cancer-secreted Gal-3 binds to CD45, activating independent pathways involving protein kinase C and ROS, resulting in sustained ERK 1/2 phosphorylation, Caspase-9 activation, cytochrome c release, and Caspase-3 activation to induce apoptosis.[109] Besides the function of Gal-3, secreted Gal-1 in the TME also correlated with increased cancer progression (following ICB therapy), which could be due to T-cell apoptosis, likely mediated through a CD45-binding dependent mechanism.[110–116] However, this correlation is not consistent across cancers. While elevated Gal-1 correlates with T-cell apoptosis in pancreatic[117] and lung[110] cancer cell lines, it was not confirmed in a melanoma cell line[108] or in vitro against activated primary T cells,[118] suggesting that its effects may be heterogeneous across malignancies.

Similarly, gangliosides and sialic acid-containing glycosphingolipids found on outer plasma membranes are overexpressed in cancers and shed into the TME.[119] Although the apoptotic effects of gangliosides and their expression in different cancers have not been investigated as extensively as galectins, Finke and Tannenbaum have elucidated their general effect on T-cell apoptosis through a series of studies. Finke *et al* demonstrate in both a GBM and a renal cell carcinoma model that cancer gangliosides are responsible for inducing T-cell apoptosis.[120 121] Moreover, Bharti and Singh show the induction of bone marrow cell apoptosis through T-cell lymphoma-derived gangliosides.[122] Regarding the mechanism of ganglioside-mediated T-cell apoptosis, gangliosides have been shown to be internalized by activated T cells, resulting in ROS production, cytochrome c release, and Caspases-8 and Caspase-9 activation.[123] This implies that gangliosides may promote both intrinsic and extrinsic apoptosis. Notably, gangliosides facilitate the intrinsic pathway of apoptosis, as evidenced by the induction of ROS, cytochrome c release, Caspase-9 activation, and downregulation of anti-apoptotic BCL genes, such as BCL-XL and BCL-2.[124]

Metabolic pathways and associated enzymes may also play important roles in T-cell apoptosis. For instance, glucose deprivation can reduce the proliferation of Jurkat cells and primary human T cells in vitro.[125] This reduction might be linked to the increase in intrinsic apoptosis since the knockdown of pro-apoptotic BH-3-only protein (ie, Noxa) improves the survival of T cells when limited glucose is available. Considering that T cells encounter significant competition in the uptake of glucose by cancer cells in TME,[126] the lack of glucose in T cells may increase the susceptibility of T cells to apoptosis, leading to impairment of the anticancer activity of T cells. In addition to the glycolytic pathway, fatty acid metabolism is critical for T cell-mediated anticancer activity. While T cells use fatty acid oxidation to form and maintain the memory phenotype,[127] inhibiting fatty acid synthase potentially reduces the expression of FasLs, preventing T cells from restimulation-induced cell death.[128] In addition to the intrinsic alteration of T-cell metabolism in inducing apoptosis, metabolites from cancer cells may also promote apoptosis in T cells. For example, kynurenine, a metabolite of tryptophan by indoleamine 2,3-dioxygenase in cancer cells, can induce apoptosis in thymocytes and terminally differentiated T helper cells.[129]

The last well-documented secretion-based methods of direct cancer-induced T-cell apoptosis are the acidic and hypoxic stress found in the TME. Acidity is caused by the 'Warburg effect', whereby cancer cells preferentially engage in aerobic glycolysis rather than oxidative phosphorylation metabolism of glucose.[130] Consequently, they increase their glucose intake to meet their energy demands, producing excess lactate acid, which is secreted into the microenvironment, causing acidification of the extracellular space.[131] Long-term exposure (>3 days) to acidic pH in the TME (pH 6.5) caused permanent damage and T-cell apoptosis in C57BL-murine B16-melanoma TILs.[132] Under extreme conditions, acidic stress (pH 3.3 for 25 min at 37°C) induces intrinsic apoptosis in Jurkat T cells by increasing cell cycle arrest.[133] Although in vitro studies demonstrated that acidic stress can alter apoptosis in T cells, the effect of acidic conditions in vivo remains unknown and requires careful validation. Along with increased acidity, hypoxia in the TME can also be a critical factor affecting T-cell apoptosis. Kiang *et al* found hypoxia-induced apoptosis in the Jurkat cell line.[134] The authors attributed apoptosis to increase NO production due to the upregulation of NO synthase, subsequently increasing Caspase-9 activation, cytochrome c levels, and Caspase-3 activation. In addition, hypoxia (1% O_2) induces apoptosis in primary T cells from healthy donors, hypothesizing it to result from a buildup of endogenous adenosine in the extracellular medium. The authors found that T cells had an upregulation of the adenosine receptor A2aR. The downstream effects of these receptors in inducing apoptosis have not been characterized.[135]

STRATEGIES TO AVOID T-CELL APOPTOSIS IN THE TME

Long-term survival and functionality of T cells are critical to ensure the anticancer efficacy of cancer immunotherapies.[136] Several strategies have been developed to prevent T-cell apoptosis. Yamamoto *et al* established a novel CAR-T cell that inhibits FasL-mediated T-cell apoptosis by truncating the intracellular death domain of Fas or introducing a point mutation (I246N) in the Fas death domain.[137] These modifications allow CAR-T cells to become resistant to cancer-induced FasL-mediated

apoptosis by inhibiting the recruitment of FADD into the apoptotic complex and preventing DISC formation. The failure of DISC formation enhanced CAR-T cell persistence and anticancer activity in a murine B16 melanoma cancer model. Importantly, Fas-engineered T cells did not show uncontrolled proliferation, at least in in-vivo models, suggesting that modulation of T-cell extrinsic apoptosis may be a safe and feasible strategy.[137] Similarly, another study reported that CRISPR-mediated KO of Fas reduced the AICD of anti-CD19 CAR-T cells during chronic exposure to target cells, which led to increased T-cell expansion.[138] In addition to modifying extrinsic apoptosis in T cells, Charo *et al* generated murine T cells that overexpress BCL-2 and tested whether this modification leads to enhanced anticancer activity of cytolytic T cells by preventing apoptosis.[35] The authors identified that BCL-2 overexpressing T cells show superior anticancer activity compared with wild-type T cells by improving long-term survival in the absence of a survival signal. Recently, another study revealed that constitutive overexpression of BCL-2 in CAR-T cells improves CAR-T cells proliferation and reduces AICD in CAR-T cells.[139] Our group further demonstrates that higher levels of BCL-2 expression in CAR-T cells of patients with lymphoma significantly correlate with enhanced clinical response (ie, CAR-T persistence and overall survival) of CAR-T therapy, suggesting that modulating intrinsic apoptosis in T cells is an important strategy to enhance CAR-T therapy.[33] In addition to BCL-2, there are other critical anti-apoptotic regulators (ie, MCL-1 and BCL-xL) affecting T-cell survival and differentiation. Studies using transgenic expression of these anti-apoptotic regulators have suggested their potential implications in T cell-based immunotherapy. For instance, constitutive expression of BCL-xL rescued activation-induced cell death of $CD8^+$ T cells in a viral infectious model.[140] Enhanced expression of MCL-1 promotes long-term memory formation in the acute phase of vaccinia virus infections.[141] Despite the beneficial effect of BCL-2 family overexpression in T cells, altering the BCL-2 signal in T cells requires additional attention, as the constitutive expression of BCL-2 in murine T cells promoted T-cell lymphoma development (ie, 18 of 68 BCL-2 transgenic mice developed T-cell lymphoma).[142]

Finally, developing strategies to avoid T-cell apoptosis would be beneficial for preventing potential apoptosis of T cells when combining immunotherapies with pro-apoptotic drugs. Our group recently reported a novel strategy to overcome venetoclax-mediated CAR-T cell toxicity by developing venetoclax-resistant CAR-T cells (ven-CAR-T).[33] In ven-CAR-T, we introduced a mutant form of BCL-2 containing a point mutation at the 104 amino acid residue (Phe104Leu or F104L) located in the binding pocket of venetoclax. Accordingly, venetoclax cannot bind to BCL-2(F104L) and loses its inhibitory function.[143 144] Therefore, by overexpressing BCL-2(F104L) in ven-CAR-T, ven-CAR-T showed strong resistance to venetoclax, leading to a significant enhancement of CAR-T cells and venetoclax combination effects.

CONCLUSIONS

As immunotherapy is ready to make its next steps and advances as a line of therapy for patients, a critical factor is the development of strategies to overcome the current limitations that preclude responses in a significant subset of patients. This review discussed the dual role of apoptosis in T cell-based immunotherapy, from cancer (ie, resistance to apoptosis) as well as a T-cell side (ie, apoptotic death).

Most cancers are characterized by resistance to apoptosis through several mechanisms, including neutralizing pro-apoptotic signals by either increasing expression of anti-apoptotic molecules such as IAPs and BCL-2 or decreasing positive regulators of the death receptor-mediated apoptosis. Therefore, increasing the sensitivity of cancer cells to apoptosis should be considered a vital strategy to improve the anticancer activity of T cell-based immunotherapies. Combining pro-apoptotic drugs may be an appealing approach for sensitizing cancer cells to T cell-mediated death; for instance, inhibiting key anti-apoptotic regulators (IAPs and BCL-2) by targeted small molecules (SMAC mimetics and ABT737) enhanced CAR-T cell-mediated anticancer activities. However, because such drugs may also induce T-cell apoptosis, careful consideration of the administration timing/dose of pro-apoptotic drugs or apoptosis-sensitizing treatments must be made to determine the optimal therapeutic regimens.

Regarding T-cell apoptosis, cancer evades immunotherapy by secreting pro-apoptotic inducers against cytolytic T cells and developing a hostile TME. Thus, there is a clear need for combinations that can prevent these evasion mechanisms. CAR-T cell therapy presents a versatile option not only for combination strategies but also for the possibility of performing genetic engineering (eg, Fas KO, mutant Fas, or constitutive overexpression of BCL-2). However, as a consequence of enhancing T-cell survival/expansion by aforementioned modulations, safety concerns such as abnormal lymphoproliferation and tumorigenesis of modified T cells appear. Therefore, it is critical to include safety switches in these models to maximize safety in clinical use (eg, the anti-inducible Caspase-9 system and antibody-mediated cellular cytotoxicity using a truncated epidermal growth factor receptor/anti-epidermal growth factor receptor antibody).

In conclusion, apoptosis is a crucial player in T cell-based immunotherapy. Deep knowledge of mechanisms of apoptosis resistance in cancer and T-cell biology is necessary to promote cancer cell apoptosis and prevent T-cell death. Several novel agents being developed together with the most recent advances in bioengineering will pave the way for the success of next-generation therapeutic combinations.

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