

Review Article

Combination of celecoxib (Celebrex®) and CD19 CAR-redirected CTL immunotherapy for the treatment of B-cell non-Hodgkin's lymphomas

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Abstract: The nonsteroidal anti-inflammatory drug (NSAID) Celecoxib (Celebrex®) received Food and Drug Administration (FDA) approval in 1998 for treatment of osteoarthritis and rheumatoid arthritis, and in recent years, its use has been extended to various types of malignancies, such as breast, colon, and urinary cancers. To maintain the survival of malignant B cells, non-Hodgkin's Lymphoma (NHL) is highly dependent on inflammatory microenvironment, and is inhibited by celecoxib. Celecoxib hinders tumor growth interacting with various apoptotic genes, such as cyclooxygenase-2 (Cox-2), B-cell lymphoma 2 (Bcl-2) family, phosphor-inositide-3 kinase/serine-threonine-specific protein kinase (PI3K/Akt), and inhibitors of apoptosis proteins (IAP) family. CD19-redirected chimeric antigen-receptor (CD19 CAR) T cell therapy has shown promise in the treatment of B cell malignancies. Considering its regulatory effect on apoptotic gene products in various tumor types, Celecoxib is a promising drug to be used in combination with CD19 CAR T cell therapy to optimize immunotherapy of NHL.

Keywords: Non-Hodgkin lymphoma, immunotherapy, CD19, chimeric antigen receptor, apoptosis, signal transduction, celecoxib, Bcl-2 family, apoptosome, resistance, mitochondria, rituximab, CHOP, adoptive cell transfer

Inflammation and cancer

Inflammation is a defense mechanism by which immune cells, such as neutrophils, monocytes, and macrophages, are mobilized to the areas that have foreign bodies due to infection or injuries. These immune cells release pro-inflammatory cytokines, a necessary physiological step in fighting infection and healing wounds. Inflammation is self-limiting in normal cells, while it remains chronic in tumor cells [1]. Several studies have shown that there is a close relationship between tumor growth and inflammation. The nuclear factor for Kappa B cells (NF-κB), a group of transcription factors related to v-Rel oncogene, is an important link between cancer and inflammation [2]. Underlying inflammation or the formation of inflammatory microenvironment caused by malignant progression, activates NF-κB. Once activated, NF-κB upregulates tumor promoting cytokines like interleukin-6 (IL-6) and tumor necrosis factors-α (TNF-α), and pro-survival proteins like

Bcl_{xL}, an antiapoptotic member of Bcl-2 family [2]. Additionally, it is proven that cell proliferation alone does not cause cancer, but an environment rich in DNA mutations, growth factors, activated stroma, and inflammatory cells is required [1]. Inflammatory signals trigger the release of several soluble factors, one of which is prostaglandin E2 (PGE₂), an enzymatic product of two Cox isoenzymes, Cox-1 and Cox-2 [3].

Physiological roles of cyclooxygenase (Cox)

Cyclooxygenase-1 (Cox-1) and Cox-2 have different physiological functions due to their differences in tissue expression and regulation [4]. Cox-1 is a house-keeping gene, constitutively expressed in almost all tissues. It produces prostaglandins (PGs) that are involved in homeostatic functions. Cox-2, encoded by the gene *Ptgs2*, is tightly regulated and is highly inducible during inflammation. It is significantly upregulated in cells with inflammatory arthritis, proinflammatory cytokines, and tumorigenic

potential [4]. With its inflammatory property, Cox-2 can promote tumor growth. Using Apc^{Δ716} knockout mice, a mouse model of human familial adenomatous polyposis (FAP), Oshima and colleagues showed that Cox-2 overexpression could induce tumorigenesis. In regular Apc^{Δ716} mice, significant amount of Cox-2 was expressed at very early stage of polyp formation. In Apc mice with *Ptgs2* knockout and Apc mice treated with Cox-2 inhibitor, MF Tricyclic, substantial decrease in polyps was noted [5]. Cox-2 catalyzes the conversion of arachidonic acid (AA) to prostaglandin endoperoxide H₂, and the reaction results in the formation of several mutagenic metabolites, such as malondialdehyde [6]. The peroxidase activity of Cox-2 can also convert xenobiotics into mutagens [6].

As mentioned above, Cox-2 produces PGE₂, a protein that facilitates tumor growth. PGE₂ induces the expression of IL-6 and haptoglobin, both of which are important regulators of angiogenesis; moreover, PGE₂ also creates an immunosuppressive environment and a tumor microenvironment that support angiogenesis [3]. Cox-2 and PGE₂ are implicated in the development of colorectal cancer [7]. Overexpression of PGE₂ can also increase the protein levels of myeloid cell leukemia-1 (Mcl-1) through a PI3K/Akt-dependent pathway in human adenocarcinoma cells [8]. Mcl-1, a member of the anti-apoptotic Bcl-2 family, is involved in the intrinsic apoptotic signaling pathway. Moreover, Cox-2 mRNA stability is regulated by p38 mitogen-activated protein kinase (MAPK), a signal transduction pathway involved in extrinsic apoptotic signaling pathway [9]. Inflammatory stimuli, such as lipopolysaccharides (LPs), IL-1, and TNF- α , activate p38 MAPK, which in turn activates Cox-2 transcription. More research is needed to determine the exact mechanisms by which Cox-2 promotes tumor growth, but from the studies conducted so far, Cox-2 most likely induces tumorigenicity not simply by its activation of carcinogens, but by interacting with other factors, mainly the apoptotic machinery.

Major apoptotic pathways

There are two main pathways the cell uses to initiate apoptosis: the extrinsic pathway and the intrinsic pathway. They are tightly regulated by antiapoptotic signal transduction pathways such as NF- κ B, PI3K/Akt, and MAPK, all of which are frequently dysregulated in tumors

[10]. The extrinsic pathway is activated by death ligands, such as TNF- α , Fas ligand (FasL), lymphotoxin, and Apo2L/TNF-related apoptosis-inducing ligand (Apo2L/TRAIL). When these ligands bind to their cognate receptors on the cell surface, Fas-associated protein with death domain (FADD) and TNFRSF1A-Associated via death domain (TRADD) are recruited to activate the initiator caspases, including pro-caspases-8, -2, and -10. The initiator caspases then activate the caspase cascade, resulting in the activation of executioner caspases. The intrinsic pathway is activated by cell stress such as chemotherapy. The cell stress triggers the cytoplasmic release of cytochrome c and second mitochondria-derived activator of caspases/direct IAP binding protein with low PI (SMAC/DIABLO) from the mitochondria, both of which are important pro-apoptotic effector proteins. Cytochrome c, together with dATP, cytoplasmic factors like Apoptotic protease activating factor-1 (Apaf-1) and pro-caspase-9, forms the large multiprotein complex called apoptosome, which then triggers the autocatalytic process and activation of caspase-9, which ultimately activates executioner caspases-3, -6, -7. Executioner caspases cleave several intracellular substrates, such as death substrate poly ADP-ribose polymerase (PARP), and trigger apoptosis. Many cases in which tumors become resistance to apoptosis are caused by down-regulation or shedding of death receptors. Death receptors, such as Fas, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) will not be expressed on the cell surface, so the death ligands cannot activate the extrinsic signaling pathway [10].

Besides the mentioned above factors, the intrinsic apoptotic signaling pathway is also regulated by Bcl-2 protein family. The pro-survival subfamily of Bcl-2 (Bcl-x_L, Bcl-w, Mcl-1, Bfl-1/A1 and Bcl-B) promotes cell survival upon exposure to cytotoxic stimuli, while the Bax-like pro-apoptotic subfamily (Bax, Bak, and Bok) and BH3-only proteins subfamily (Bik, Bad, Bid, Bim, Bmf, Hrk, Noxa and Puma) promote cell death [11]. Whether the cell undergoes apoptosis depends on the balance between the pro-survival and pro-death signals from these three subfamilies. BH3-only proteins subfamily serves as cellular stress sensor. Upon receiving cellular stress, BH3-only proteins inactivate Bcl-2 like proteins, resulting in the activation of Bax-like proteins. Activated Bax-like proteins

permeabilize the outer mitochondrial membrane, triggering cytochrome c release and initiating the intrinsic pathway leading to apoptosis. Loss of Bax function and reduced levels of Bax are linked to resistance to chemotherapy and poor prognosis in pancreatic and ovarian cancer cells [12].

There are other proteins involved in the development of apoptosis-resistant tumor cells. Hypoxia inducible factor-1 (HIF-1) is a key regulator of hypoxia, a process that creates environmental stress and induces apoptosis. However, tumor cells, after several periods of hypoxia, adapt to the environmental stress and become resistant to apoptosis. HIF-1 can trigger hypoxia-mediated apoptosis by increasing the level of BCL2/Adenovirus E1B 19 kDa Interacting Protein 3 (BNIP3) and its homologue NIX, which in turn inhibit the pro-survival effect of Bcl-2; however, HIF-1 can also prevent apoptosis by inducing the expression of IAP-2 [13]. HIF-1 is an important apoptosis mediator, and cancer cells seem to selectively use HIF-1 to avoid undergoing hypoxia-mediated apoptosis.

Besides IAP-2, there are other proteins within the IAPs family that are important in intrinsic apoptotic signaling pathway, namely cellular inhibitors of apoptosis (cIAP)-1, cIAP-2, X-linked inhibitors of apoptosis protein (XIAP), and survivin. Under normal conditions, these proteins inhibit the activation of executioner caspases. SMAC/DIABLO, upon activation, physically associates with these proteins and removes the inhibitors of caspase activation.

Celecoxib as a selective Cox-2 inhibitor

As mentioned above, Cox-2 expression increases during inflammation. Therefore, Celecoxib, with its anti-inflammatory property, is theoretically a novel drug for cancer treatment. Celecoxib inhibits Cox-2 by interfering with prostaglandin-mediated upregulation of anti-apoptotic proteins such as Mcl-1 [14]. In Cox-2 overexpressed cells (Cox-2/cl.4), treatment of cytotoxic dose of 10 μ M celecoxib and 25 μ M NS-398, another Cox-2 inhibitor, significantly reduces the level of Mcl-1 [8]. Moreover, under the same experimental conditions, both inhibitors can inhibit PGE₂ by 70-80% [8]. Celecoxib hinders Cox-2 activities in several cell lines. It exerts antiproliferative effects on Raji and Ramos Burkitt lymphomas *in vitro* [15] and in nude mice having intracranial lymphomas,

which mimic human central nervous system (CNS) lymphomas [16]. Celecoxib is also an effective apoptotic inducer of B cells lymphoma, but not necessarily of T cells lymphoma [17].

In a study using cells of hemopoietic origin, treatment with high doses of celecoxib was very effective in patients with multiple myeloma (MM) [18]. More than 30% of malignant cells in MM had overexpression of Cox-2. Patients with MM tend to develop resistance to chemotherapy, so celecoxib is a good alternative therapeutic drug. In a phase II clinical trial, patients with relapsed and refractory MM were given thalidomide with celecoxib at doses ranging from 200 to 800 mg/day [19]. The results were promising: those who took doses greater than 400 mg/day had greater progression-free survival than those who took doses equal to or less than 400 mg/day (12.7 months compared to 4.6 months). Patients who took higher doses also had a better overall survival rate (OSR) than those who took the lesser dose (29.6 months compared to 18.9 months). However, adverse effects (AEs), such as peripheral edema and renal complication, were observed in some patients [19].

Celecoxib is also an effective drug to use in treatment of patients with NHL. In a phase II study, 35 patients with relapsed or refractory NHL were treated with high doses of celecoxib (400 mg p.o.bi.d) [20]. The median progression-free rate was 4.7 months and median overall survival rate was 14.4 months with 8.4 months median follow-up. Even though celecoxib was used in high doses, the AEs observed were minimal. Gastrointestinal toxicity was observed with no interference with compliance. Most AEs were grade 1 and 2, including nausea, hypertension, and fatigue. Pharmacokinetics data showed that celecoxib was stable for a prolonged period. Per a preclinical model of Kerbel and colleagues, a plasma concentration having more than 500 μ g/L was antiangiogenic [21]. The plasma concentrations were taken during the 12-hour period after the administration of the first dose of celecoxib. After a single dose of 400 mg, the peak concentration (C_{max}) was $2,369 \pm 1,586$ μ g/L at a median time of 3.2 hours, while C_{min} after a single dose was 539 ± 335 μ g/L. Additionally, celecoxib has an apparent clearance (Cl/F) of 0.6 ± 0.4 L/h/kg and an elimination half-life ($t_{1/2}$) of 4.1 ± 0.9

Table 1. Summary of clinical data using Celebrex in various tumors

Tumor Type	Dose	Overall Response Rates (ORR) (%)	Overall Survival (OS) (months)	Reference	Clinical Phase
Colorectal	2×200 mg twice per day of Celebrex, in combination with FOLFIRI**	32	19.9	Kohne, 2007	
	2×200 mg twice per day of Celebrex, in combination with CAPIRI***	22	14.75	Kohne, 2007	
Multiple Myeloma	Median dose of 400 mg thalidomide/day and 800 mg celecoxib/day	42	21.4	Prince, 2005	II
	Median dose of 400 mg thalidomide/day	29	19.8	Prince, 2005	II
NSLC	Celecoxib 400 mg p.o. twice/day with docetaxel initiated at a dose of 75 mg/m ²	34	6.3	Csiki, 2005	II
NSLC	Increasing doses of celecoxib (200 to 800 mg) twice per day with a fixed dose of erlotinib	33		Reckamp, 2006	I
NSCLC*	Palliative radiation (total dose: 45 Gy) 3 Gy/fraction/day in 3 weeks with 200-800 mg of celecoxib	34.1		Liao, 2005	I
	Definitive radiation therapy (total dose: 66 Gy) 2 Gy/fraction/day in 6.5 weeks with 200-800 mg of celecoxib	31.7		Liao, 2005	I
	Total radiation dose of 63 Gy at 1.8 Gy/fraction/day in 7 weeks after induction chemotherapy with 200-800 mg celecoxib	24.4		Liao, 2005	I
Pancreatic Cancer	Gemcitabine 1,000 mg/m ² was given as a 30-min intravenous (IV) infusion on days 1 and 8, every 3 weeks; celecoxib was administered orally in addition to gemcitabine (400 mg twice per day)	9	9.1	Ferrari, 2006	II

*NSCLC Cancer: Non-Small Cell Lung Cancer. **FOLFIRI: irinotecan (180 mg/m² i.v. on days 1, 15 and 22); folinic acid (200 mg/m² i.v. on days 1, 2, 15, 16, 29 and 30); 5-fluorouracil (400 mg/m² i.v. bolus, then 22-h, 600 mg/m² infusion). ***CAPIRI: irinotecan (250 mg/m² i.v. infusion on days 1 and 22); capecitabine p.o. (1000 mg/m² b.i.d. on days 1-15 and 22-36).

hours [22]. Celebrex is being clinically used in various tumor models (summarized in **Table 1**).

The pro-apoptotic effect of celecoxib does not depend entirely on Cox-2 inhibition. Several studies have shown that celecoxib can also induce apoptosis in Cox-2 negative cells. Celecoxib showed substantial antiproliferative effects on epithelial cancer cell lines, which had no detectable levels of Cox-2 expression [23]. In a study by Song and colleagues, Cox-2 depletion did not induce cell death and some of celecoxib derivatives that did not have Cox-2 inhibitory activity could facilitate apoptosis [24]. Interestingly, there are reports on the inhibition of cell proliferation in *in vitro* and *in vivo* models of Burkitt's lymphoma due to downregulation of cyclins A and B and the loss of cyclin-dependent kinase (CDK) activity upon treatment with dimethyl-celecoxib (DMC), a celecoxib analog that lacks Cox-2 inhibitory function [15]. Therefore, Cox-2 presence in the cell is not required for celecoxib pro-apoptotic effect.

Modulation of apoptotic machinery by celecoxib

Celecoxib induces apoptosis via the intrinsic signaling pathway. The apoptotic effect is

Bcl-2-independent and apoptosome-dependent. In Jurkat cells, Apaf-1 and pro-caspase-9 were required for celecoxib-induced apoptosis, while the presence or absence of Bcl-2 did not interfere with celecoxib-induced apoptosis [25]. Overexpression of Bcl-2 did not affect the effectiveness of celecoxib in Jurkat cells and Bcl-2 expression levels were not modified by celecoxib, as seen by the unaltered size and abundance of nonphosphorylated Bcl-2 protein levels. Additionally, Bcl_{-XL} lacks significant inhibitory effects on celecoxib-induced apoptosis.

In lymphomas, arachidonic acid is converted to prostaglandins by Cox-2, leading to the upregulation of several anti-apoptotic proteins such as Bcl-2, PI3K/Akt, and Mcl-1 [26]. Surprisingly, only Mcl-1 and Bcl_{-XL}, not Bcl-2 or PI3K/Akt, form a high affinity complex with Bak, thus blocking apoptosis [27]. Cytotoxic signals activate BH3-only proteins, which interact with Mcl-1 and Bcl_{-XL}, thus displacing Bax and apoptosis ensues. Both Mcl-1 and Bcl_{-XL} are required to inhibit Bak pro-apoptotic activity; when Mcl-1 and Bcl_{-XL} do not bind Bak, apoptosis is induced. Celecoxib interferes with pro-survival signals by downregulating Mcl-1. In Jurkat T lymphoma cells treated with celecoxib, there was a sharp

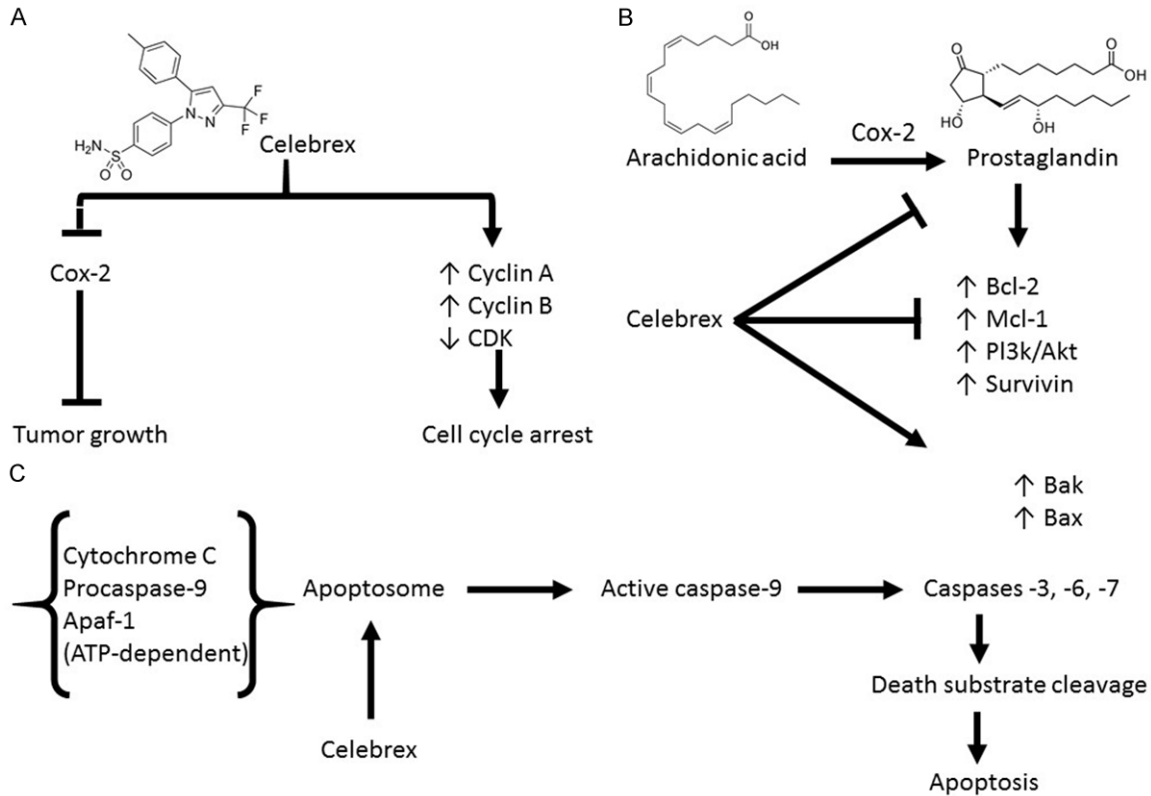


Figure 1. Potential mechanisms of celebrex-mediated apoptosis. A. Either through direct cox-2 inhibition, or via induction of cyclins A, B and inhibition of cyclin-dependent kinase (CDK) activity, Celebrex inhibits tumor growth and induces cell cycle arrest. B. Cox-2 mediates the conversion of arachidonic acid to prostaglandin, which upregulates the expression of anti-apoptotic proteins Bcl-2, Bcl_{xL}, Mcl-1, survivin as well as increasing the activity of the PI3K/AKT anti-apoptotic signal transduction pathway. These events confer a growth advantage to the tumor cells. Celebrex, via cox-2 inhibition, interferes with the generation of prostaglandin resulting in down-regulation of anti-apoptotic genes and concurrent induction of pro-apoptotic Bax and Bak; facilitating the generation of a pro-apoptotic environment. C. In a cox-2-independent, apoptosome-dependent manner, Celebrex potentiates the formation of multi-protein complex apoptosome and caspase-9 processing, which in turn leads to processing and activation of executioner caspases-3, -6, -7, cleavage of death substrates and apoptosis.

decline of Mcl-1, allowing Bak to trigger apoptosis. An abundance of Bcl_{xL} compensates for the function of Mcl-1 and blocked apoptosis in Mcl-1-negative cells [28]. The presence of Bak is more crucial than the presence of Bcl-2 for celecoxib-induced apoptosis to proceed. In fact, Jurkat cells that were Bak/Bax-negative showed nearly complete resistance to celecoxib-induced apoptosis, while overexpression of Bcl-2 only had limited anti-apoptotic effects [29]. Overall, the mechanism through which celecoxib induces apoptosis in intrinsic signaling pathway requires the presence of functional Bak. In human T-cell leukemia virus type 1 (HTLV-I), celecoxib blocks Akt/GSK3 β survival signaling pathway to induce apoptosis via the intrinsic pathway that is accomplished by acti-

vating Bax and inhibiting the PI3K/Akt signaling pathway. This is associated with malignant transformation [30]. Possible mechanisms of Celebrex-mediated apoptosis are depicted in **Figure 1**.

Celecoxib interacts with other factors to induce apoptosis

Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are the etiological agents involved in aggressive NHL. PGE₂ affects the eicosonoid (EP) receptors, especially EP1 and EP4, blocking apoptosis. By using Cox-2 inhibitor in combination with EP receptors antagonists, significant apoptosis was induced in EBV and KSHV-positive cells [31].

Cytotoxic signals that cause cells to undergo apoptosis are also activated by endorecticulum (ER)-stress. In Raji cells, the apoptotic effects of celecoxib could be improved in combination with bortezomib, the proteasome inhibitor that is also capable of inducing ER-stress [32].

In acute myeloid leukemia (AML), celecoxib and doxorubicin drastically reduced cell proliferation and increased apoptosis [33]. Downregulation of cyclin E and CDK-2, both of which are key regulators of cell cycle progression, was observed. The pro-apoptotic effect was also linked to G0/G1 phase cell arrest and survivin downregulation. Survivin, a multifunctional member of IAP family, interferes with caspases activation to suppress apoptosis [14]. In the presence of celecoxib and DMC, reduced survivin level prevents tumor growth more effectively. Survivin is potentially a Cox-2 independent target of celecoxib (**Figure 1**).

Immunotherapy for the treatment of non-Hodgkin's lymphoma (NHL)

Several patients with NHL have undergone a novel immunotherapy modality that utilizes chimeric antigen receptors (CARs). CARs are fusion proteins that have both T-cell activation domain and antigen recognition moieties [34]. By genetically modifying T cells to express CARs, these T cells can specifically recognize specific surface markers such as CD19, a protein that is only expressed in B-cell lineages and not on hematopoietic stem cells, thus effectively targeting CD19⁺ NHL. In one clinical trial, 4 of the 8 patients who had an infusion of anti-CD19-CAR-transduced T cells and a course of IL-2 in combination with chemotherapy exhibited long-term depletion of normal polyclonal CD19⁺ B-lineage cells, CD19 CAR T cells were detected in the blood of all patients [34]. However, like chemotherapy, tumors develop resistance through inherent or acquired anti-apoptotic mechanisms. New approaches are necessary to overcome this issue, and the use of celecoxib in treatment is a promising one.

Adoptive T cell therapy as an alternative approach to immunotherapy

Among all recent immune-based therapeutic strategies, adoptive T cell (ATC) therapy is a powerful tool that has promising potential in eradicating apoptosis-resistant tumor cells.

Tumor-reactive lymphocytes are selected *ex vivo* and then adoptively transferred into patients. These lymphocytes are often administered with growth factors to enhance their survival, expansion, and cytotoxic potential *in vivo* [35]. In ATC using tumor-infiltrating lymphocytes (TILs), T cells are isolated from fresh biopsy specimens obtained from patients and tumor-specific T cells are selected and expanded using high levels of interleukin-2 (IL-2) [36]. Besides TIL-based ATC, in recent years, genetically modified T cells, such as T cell receptor (TCR) modified and chimeric antigen receptor (CAR) T cells [37], have gained great interest. These modified T cells have enhanced anti-tumor effects and higher specificity for tumor cells compared to regular T cells, thus improving the efficacy of the immune system of immunosuppressed patients.

T cell receptor (TCR) transgenic T cells' mode of action and limitation

For TCR-engineered T cells, the desired TCR that is specific to a particular tumor associated antigen (TAA) is transferred to T cells via genetic means. When genes encoding TCR α and β chains are transferred into peripheral blood T lymphocytes, the antigen-specific recognition property of these lymphocytes is enhanced significantly [38]. T cells with highly expressed TCRs for MART-1 and gp100 antigens, both of which are expressed on melanomas and melanocytes, are more reactive to metastatic melanoma than regular T cells [39]. Inducing T cell immunity using TCR transgenic T cells is an appealing immunotherapeutic approach, but it has its own limitations. After prolonged exposure to transduced T cells, tumor cells undergo a change in which they express less or no antigens that these T cells can bind to, rendering the treatment ineffective. Administration of transduced T cells is limited to vaccination, which decreases the optimal response in patients with immunodeficiency [40]. Moreover, tumor recognition by TCR-engineered T cells is human leukocyte antigen (HLA)-dependent, making the induction of T cells immunity difficult. HLA is the human version of the major histocompatibility complex (MHC), a group of genes that encodes cell surface proteins to help lymphocytes distinguish host cells from foreign substances (self-recognition and non-self-rec-

ognition). Each individual has a specific MHC haplotype. If the transduced T cells derived from an individual that has an incompatible MHC haplotype with the recipient (MHC mismatch), then induction of immunity might not be successful [40].

Chimeric antigen receptor (CAR) T cells: structures and development

Unlike TCR-engineered T cells, CAR-engineered T cells recognize tumor cells in an HLA independent fashion. CAR T cells allow the use of a variety of different combination of signaling and costimulatory domains for optimal T cell recognition and activation. A CAR typically has a ligand-binding domain, such as a single-chain variable fragment (scFv) derived from a monoclonal antibody or an antigen-binding fragment (Fab), and a signaling domain, which usually has CD3 ζ , a component of the TCR complex [41, 42]. Antigen recognition mediated by scFv allows CAR-T cells to recognize their target independently of MHC [43]. CD3 ζ serves as an activation domain. T cells activation is promoted by the phosphorylation of the tyrosines in immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 ζ ; the first and third ITAMs are linked to apoptosis [44].

Three generations of CAR-engineered T cells have been created so far. The first generation consists of only a ligand-binding domain and signaling domain without any co-stimulation [45]. The second and third generation have different co-stimulatory domains that help enhance the specificity of T cells as well as other effector functions such as proliferation and cytokine production [46]. In second generation CAR-T cells, the activation domain is fused with the co-stimulatory domain, which can be CD28, 4-1BB, OX40 or DAP10 [41]. Dual-signaling CAR-T cells help enhance the strength of signaling and the persistence of transduced T cells in the body [47]. Third generation CAR T cells have a second costimulatory domain added to the primary costimulatory domain that is used in the second generation. The additional costimulatory domain enhances the cytotoxic potential and effector functions of T cells, including proliferation, expansion and cytokine production, against tumors [48]. A common example of a third-generation CAR T cell would be CD28/4-1BB/CD3 ζ [49].

Chimeric antigen receptor (CAR)-transduced T cell therapy for NHL

Lymphoma is a cancer caused by malignant lymphocytes. It has two subtypes: Hodgkin lymphoma (HL) and NHL. They differ in gene expression profiles. HL is commonly found as nodular sclerosis with high level of Hodgkin Reed-Sternberg (HRS) cells [50]. It is linked to primary mediastinal B cell lymphoma (PMBL), since both have amplified *JAK2* gene expression [51]. Compared to HL, NHL is much more prevalent, accounting for 90% of lymphoma cases [52]. NHL has several subtypes, 85% of which arises from malignant B cells and the others arise from malignant T cells and natural killer (NK) lymphoma [52]. The subtypes of NHL are classified based on the stage of B cell differentiation that they are derived from and the type of protooncogenes that they expressed. There have been several revisions of NHL classification, but the generally accepted subtypes are: lymphoplasmacytic lymphoma, follicular lymphoma, mantle-cell lymphoma, marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) lymphoma, diffuse large B-cell lymphoma, Burkitt's lymphoma, and anaplastic large T-cell lymphoma [53-55]. Most of these lymphomas are of B cell origin, making them ideal targets for treatment using CAR-transduced T cells.

Anti-CD19 CAR-engineered T cell therapy for NHL

Among several CAR-transduced T cells designed so far, anti-CD19 CAR T cells are especially effective against B cells malignancies [42]. Anti-CD19 CAR-transduced T cells therapy is an appealing alternative to rituximab and CHOP in the treatment of NHL. CHOP is a first generation combination chemotherapy comprised of cyclophosphamide, doxorubicin, vincristine, and prednisone used for diffuse large-B-cell lymphoma [56]. It only induces complete response in 40% of elderly patients and the overall survival rate is only 35% [57]. CHOP is very toxic for elderly patients, but if given reduced CHOP regimens, the treatment would not be as effective [58]. Several attempts to increase the efficacy of CHOP have been made, and one of the most effective strategies is combining CHOP with rituximab, a chimeric anti-CD20 IgG1 monoclonal antibody. Rituximab binds

specifically to CD20, an antigen expressed in 90% of B cell lymphomas [59]. It is quite effective when given as a single-agent to treat patients with indolent lymphoma [60]. In one study, a complete response of 76% was achieved in patients treated with CHOP and rituximab compared to a complete response of 63% in patients treated with CHOP alone [61]. CHOP plus rituximab (R-CHOP) treatment is also very effective in younger patients. Two groups of 18 to 60 years old were given either CHOP or CHOP plus rituximab [62]. The group given both CHOP and rituximab had a 79% of 3-year event-free survival compared to 59% of 3-year event-free survival in group given only CHOP [62]. Although R-CHOP has better efficacy than treatment with CHOP alone, tumor cells eventually develop resistance to this treatment in a similar fashion to other types of chemotherapy. To overcome this challenge, anti-CD19 CAR-engineered T cells were developed to make use of the immune system, reducing the severity of AEs due to toxic chemicals and increasing TAA specificity.

CD19 is an ideal antigen for immunotherapy, since it is expressed only on B-cell leukemia, lymphomas, and normal B cells, but not on other types of cells [63, 64]. Anti-CD19 CAR T cells can target B-cell leukemia and lymphomas specifically without inducing apoptosis in other cell types, giving fewer side effects compared to other types of CAR-T cells that target more ubiquitous antigens. Gene that codes for anti-CD19 is put into scFv and the activation domain can be either CD28/CD3 ζ or 4-1BB/CD3 ζ . The costimulatory molecule CD28 is required for T cell activation and survival. It binds to B7.1 (CD80) and B7.2 (CD86) on tumor cells to trigger apoptosis [65]. In one trial, 5 patients with B-cell acute lymphoid leukemia (ALL) were treated with CD28-containing CD19-CAR T cells [66]. After lymphodepletion and CAR-T cell infusion, all patients achieved complete remission.

4-1BB, a member of TNF receptor superfamily, has a high affinity for 4-1BBL, a ligand that is expressed on activated macrophages and B cells [67]. A clinical trial using antiCD19scFv/4-1BB/CD3 ζ to treat children with relapsed or refractory CD19⁺ ALL [68]. The overall survival (OS) was 78% and the persistence of CTL019 (CAR targeting CD19) cells continued for 1 to 26 months after infusion. In another clinical trial, out of the 8 patients with B cell lympho-

mas, 4 patients receiving an infusion of anti-CD19 CAR-T cells (antiCD19scFv/4-1BB/CD3 ζ) and a course of IL-2 in combination with chemotherapy had long-term depletion of normal polyclonal CD19⁺ B-lineage cells [34]. Different levels of anti-CD19 CAR gene could be detected in the blood of all patients. For patient 1 to patient 6, the gene was detected within 20 days of initial infusion, while it was detected after 14 weeks for patient 7 and 8 weeks for patient 8 [34].

Mechanisms by which tumor cells avoid recognition by CAR T cells

Downregulation of CD19 expression on tumor cell surface

Treatment using CAR T cells has great potential, but there are some limitations that needed to be overcome. Since anti-CD19 CAR T cells mode of action relies heavily on the antigen recognition provided by the binding of anti-CD19 scFv on T cells to CD19 receptors on tumor cells, tumor cells can avoid anti-CD19 CAR T cells by downregulating or inhibiting CD19 expression. For example, the expression of C/EBP α and C/EBP β in differentiated B cells will efficiently reprogram the cells into macrophages [69]. C/EBPs inhibits Pax5, a transcription factor of B cells, causing downregulation of CD19. Pax5 gene codes for several B-cell activator proteins (BSAPs), which are expressed exclusively in B-lymphoid lineage cells [70]. Therefore, downregulation of Pax5 will ultimately result in CD19 downregulation. In a timed course analysis, IL-7 enhanced the expression of CD19 on the surface of progenitor B lineage cells originated from human bone marrow [71]. In one study, patients with antibody-deficiency syndrome were found to have low level of CD19 expression due to a homozygous mutation in the CD19 gene despite having normal level of B cells [72]. In another study, immunodeficient patients who had normal amount of CD19 alleles but defective CD81 gene also had low level of CD19 expression [73]. In these cases, CD19 gene sequence remains intact, yet the cells lack the CD19 antigenic epitope, possibly caused by mutations in mRNA splicing [74].

Alternative B cell differentiation pathways

One patient with CLL undergoing anti-CD19 CAR-transduced T cells was reported to have

mature lymphoma that could avoid T cells recognition by differentiating in a pathway different from that of its normal counterpart [75]. Prior to receiving anti-CD19 CAR-engineered T cells therapy, the patient was shown to already have partial loss of CD19 and other B cell markers by flow cytometry. This suggested that B cell differentiated abnormally before anti-CD19 CAR T cells were administered. During the course of treatment, CLL transformed to plasmablastic lymphoma (PBL) and CD19⁺ leukemia. PBL had a mutation in complementarity-determining region 1 (CDR1), rendering the immunoglobulin heavy chain (IGH) reading frame unproductive. TP52 sequencing revealed a p.Gly245Ser in PBL [75].

Normal mature B cell repertoire requires the engagement of B cell activating factor receptor (BAFF-R) by BAFF [76]. CD19⁺ cells also have BAFF expression, but low or no mBAFF, which is expressed at a higher level in NHL macrophages than in healthy macrophages. The elevated level of BAFF might be linked to tumor B cell differentiation pathway that results in apoptosis triggered by CD19 antigen recognition.

Aberrant B cell antigen receptor (BCR) signaling pathways

B-cell antigen receptor (BCR) signaling pathways are highly regulated in normal B cells, but in NHL, they are aberrantly activated [77]. Many proteins involved in these pathways are activated by CD19. BCR signaling requires activation of protein tyrosine kinase (PTK). CD19 and Src family PTKs undergo several phosphorylations, creating an amplification loop that greatly enhances B cell activation upon CD19 engagement [78]. Lyn, a member of Src family PTK and initiator of BCR signaling, is recruited by CD19 [79, 80]. When BCR is activated, Lyn phosphorylates Vav and some residues of CD19 [80]. The activated Vav in turn initiates mitogen-activated protein kinase (MAPK) pathways. In malignant B cells, MAPKs, namely extracellular signal-regulated kinase (ERK) and p38, and Lyn are constitutively activated [81].

CD19 also initiates phosphoinositide-3-kinase (PI3K)/Akt signaling pathway. It activates PI3K by binding to the regulatory subunit p85 of PI3K [82]. PI3K in turn activates Akt downstream via phosphorylation, leading to activation of mTOR, the mammalian target of rapamycin [83]. In

primary effusion lymphoma (PEL), a subtype of NHL, PI3K/Akt/mTOR pathway is constitutively active and dual inhibition of both PI3K and mTOR effectively inhibits tumor proliferation [84].

Bcl-2, a family of regulatory proteins, is commonly overexpressed in NHL, leading to resistance to apoptosis and promotion of tumorigenesis [85]. Over 40% of patients with diffuse large B cell lymphoma were reported to have high level of Bcl-2 expression [86]. Bcl-2 family consists of both pro-apoptotic and anti-apoptotic proteins. In most tumor types, the anti-apoptotic members of Bcl-2 tend to be overexpressed. Out of eight leukemia or lymphoma cell lines, seven were shown to have high levels of anti-apoptotic Bcl-2 proteins, especially Bfl-1, Mcl-1, and Bcl-x_L [87]. Many different signaling pathways were potentially responsible for promoting Bcl-2 expression. Elevated levels of insulin-like growth factor-1 (IGF-1) were observed in malignant effusions, making it a possible marker for solid tumors [88]. When its receptor, IGF-1R, a receptor tyrosine kinase (RTK), is inhibited, tumor progression in chronic lymphocytic leukemia (CLL) is limited [89]. IGF-1 induces Bcl-2 promoter containing cAMP-response element (cAMP) site via cAMP-binding protein (CREB) signaling pathway [90]. Akt signaling was also found to play a role in this pathway. Cell lines expressing Akt showed increased CREB activity, which resulted in higher levels of Bcl-2 expression [91]. It is possible that by inhibiting Akt, NHL would become responsive again to apoptotic signals from the immune system or chemotherapy, as this method worked in a study using pancreatic tumor cell lines [92].

Aberrant expression of Bcl-2 family proteins is linked with drug resistance in various types of cancer [93]. In MCF-7 human breast cancer cells, estrogen induces the expression of Bcl-2 proto-oncogene transcripts significantly, leading to resistance to Adriamycin, a chemotherapy drug [94]. Lymphomas that have prolonged exposure to rituximab, chimeric anti-CD20 monoclonal antibody, become unresponsive to both rituximab and the chemotherapy drugs that rituximab is used with. Rituximab was not effective in chemosensitizing rituximab-resistant (RR) clones, developed from lymphoma lines, potentially due to hyperactivation of NF-κB and ERK1/2 pathways, resulting in over-

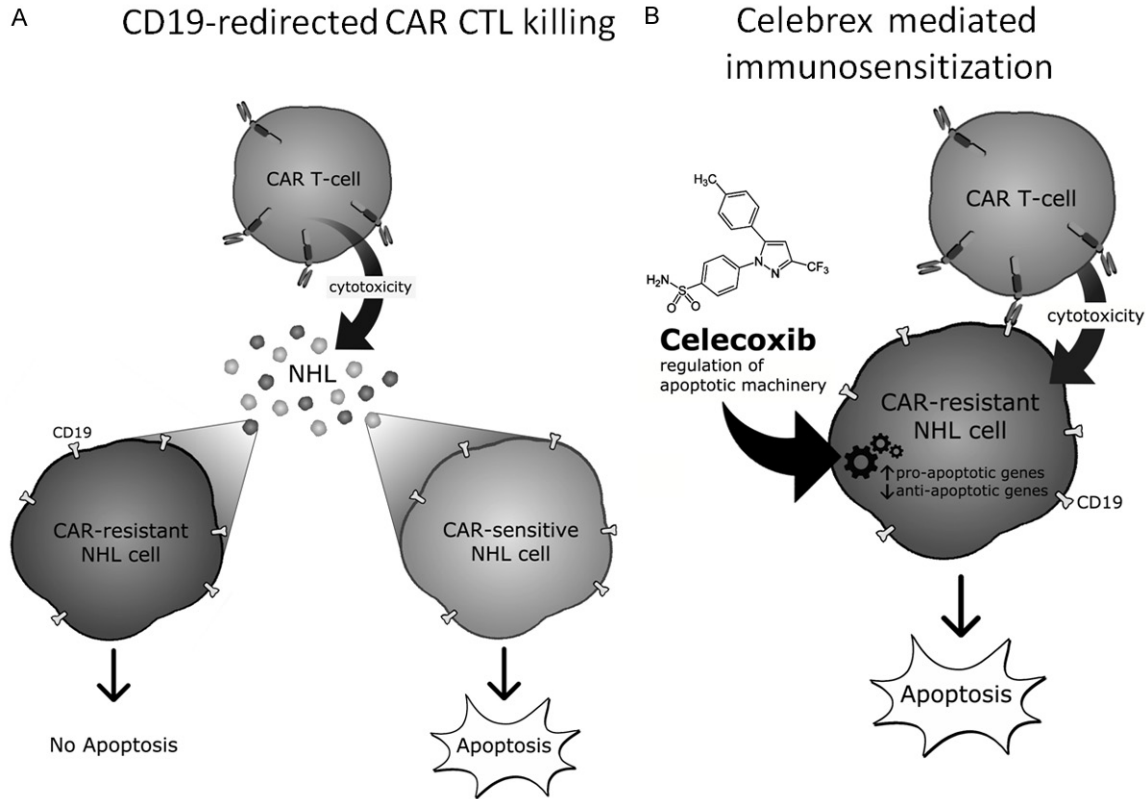


Figure 2. CD19-redirection CAR T cell therapy of NHL. A. CD19-redirection CAR T cells can induce apoptotic cell death in CD19⁺ sensitive NHL cells, while those tumor cells with distorted apoptotic machinery exhibit resistance despite adequate surface CD19 expression. B. The anti-inflammatory drug Celecoxib® (Celecoxib) regulates the expression profile of apoptotic gene products, favoring the generation of a pro-apoptotic milieu; thus, CD19 CAR T cell-resistant NHL cells undergo apoptosis.

expression of Bcl-2 and Mcl-1 in these clones [95]. Bcl-2 proteins are capable of enhancing drug resistance in cancer cells mainly due to their anti-apoptotic properties. By blocking Bax and Bak, which are pro-apoptotic proteins of Bcl-2 family, overexpressed anti-apoptotic Bcl-2 proteins (Bcl-2, Mcl-2, and Bcl-x_L) inhibit the intrinsic apoptotic machinery of the cell [96]. Since anti-CD19 CAR therapy works by inducing both extrinsic and intrinsic apoptotic pathways, overexpression of anti-apoptotic Bcl-2 proteins might render the treatment ineffective. Using Bcl-2 family inhibitors, such as ABT-737, in combination with anti-CD19 CAR therapy can overcome this problem, increasing the efficacy of CAR T cells and managing tumor growth [97].

A study using CD19^{-/-} mice demonstrated that CD19 propagated BCR-induced survival signals [82]. In clinical studies, mutated CD19 is linked to autoimmune diseases. A patient with a mutation in the splice acceptor site of intron 5 of maternal allele of CD19 had hypogammaglo-

binemia and no detectable antibodies against measles, rubella, tetanus and pertussis toxin, even though he was vaccinated [98]. In addition to BCR-dependent signaling pathways, CD19 is also capable of initiating MYC-driven lymphomagenesis [99]. Malignant B cell lymphomas have elevated levels of c-MYC, which in turn is greatly enhanced by CD19; therefore, by inhibiting CD19, the oncogenic capabilities of c-MYC is limited [100].

With CD19 as the upstream activator of several BCR-dependent signaling pathways, using anti-CD19 CAR therapy seems to be a promising therapeutic approach, as it will prevent the transient association of CD19 to BCR, thus inhibiting subsequent BCR-dependent signaling pathways that promote proliferation in NHL. Moreover, since CD19 is also involved in BCR-independent pathways, anti-CD19 CAR therapy potentially has more applications than previously reported.

Immunosuppressive microenvironment

The immunosuppressive microenvironment in which these cells grow is also an obstacle in using CAR T cells for cancer treatment. Tumor microenvironment has a high number of cytokines and immunosuppressive growth factors, including vascular endothelial growth factor (VEGF), interleukin (IL)-10, and transforming growth factor (TGF)- β [101]. These cytokines impede the anti-tumor activity of T cells, thus lowering the efficacy of CAR T cells. Before CAR T cells are administered, lymphodepletion is conducted. However, lymphodepletion might exacerbate the immunodeficiency that exists in patients, resulting in severe AEs caused by opportunistic diseases.

Anti-CD19 CAR T cells therapy has a larger range of cell depletion than anti-CD20 therapy, since it also affects pro-B cells and plasma cells (PCs) [102]. Anti-CD19 therapy works in a manner similar to anti-CD20 therapy, so it is possible that anti-CD19 therapy has the same drawbacks as anti-CD20 therapy. When treated with rituximab, a majority of patients had significantly impaired response to serological antibodies [103, 104]. A possible way to mediate this problem is to supply patients with Ig antibodies via intravenous infusion [34], although the effectiveness of this approach remains unclear.

Another problem with CAR T cells therapy is that engineered T cells can persist in the body longer than the time they are designed to be. CAR T cells can cause several AEs such as fevers, hypotension, hypoxia, and neurologic changes [34, 66, 68], so the timing and doses of CAR T cells infusion need to be optimized to avoid tumor resistance to CAR T cells, high grade AEs, and exacerbated immunodeficiency.

Conclusions and future directions

The development and selective outgrowth of apoptosis-resistant tumor cells is a major hurdle in successful cancer therapy. Aberrant apoptotic machinery culminates in tumor cells that develop cross-resistance to a wide array of structurally and functionally distinct anti-cancer agents. Therefore, it is empirical to design novel approaches to modulate apoptotic machinery in order to bypass tumor resistance.

Among new therapeutic drugs developed in recent years, celecoxib is a promising alternative. Its mechanism of action is flexible: it induces apoptosis in the presence or absence of Cox-2 via intrinsic signaling pathway in a Bcl-2 independent and apoptosome-dependent manner. Additionally, several studies have shown that celecoxib further enhances apoptosis of tumor cells with minimal AEs when used in combination with other drugs, such as bortezomib, doxorubicin, and thalidomide.

Recent modern developments in utilization of the immune system to harness NHL suggest a promising role of CD19 CAR T cell therapy in NHL. However, a subset of tumor cells either inherently resistant or develop resistance to CAR-mediated immunotherapy. Based on the apoptotic gene regulatory effects of celecoxib, we propose that combination of CD19 CAR T cell therapy and celecoxib can potentially improve the treatment outcome of NHL patients (**Figure 2**).

Future research is warranted to understand the details of induction of apoptosis by celecoxib in various tumor models. This insight will allow more generalized, optimized, and effective treatments for patients. It will also help researchers expand the possible usage of celecoxib in combination with other anti-cancer modalities, such as histone deacetylase inhibitors (HDACi) in immunotherapy.

Disclosure of conflict of interest

None.

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