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Short survey

Cytokines for the induction of antitumor effectors: The paradigm of Cytokine-Induced Killer (CIK) cells

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ABSTRACT

Cytokine-Induced killer (CIK) cells are raising growing interest in cellular antitumor therapy, as they can be easily expanded with a straightforward and inexpensive protocol, and are safe requiring only GMP-grade cytokines to obtain very high amounts of cytotoxic cells. CIK cells do not need antigen-specific stimuli to be activated and proliferate, as they recognize and destroy tumor cells in an HLA-independent fashion through the engagement of NKG2D. In several preclinical studies and clinical trials, CIK cells showed a reduced alloreactivity compared to conventional T cells, even when challenged across HLA-barriers; only in a few patients, a mild GVHD occurred after treatment with allogeneic CIK cells. Additionally, their antitumor activity can be redirected and further improved with chimeric antigen receptors, clinical-grade monoclonal antibodies or immune checkpoint inhibitors. The evidence obtained from a growing body of literature support CIK cells as a very promising cell population for adoptive immunotherapy. In this review, all these aspects will be addressed with a particular emphasis on the role of the cytokines involved in CIK cell generation, expansion and functionalization.

1. Introduction

Adoptive cell therapy (ACT) aims at restoring cancer recognition by the immune system, leading to effective tumor cell killing. ACT is based on the administration of antitumor immune cells, which have been stimulated and expanded *ex vivo* to obtain highly active tumor-specific effectors to be finally transferred back to the patients. If required, these activated cells can also be genetically modified to express tumor-specific recognition molecules, such as chimeric antigen receptors (CAR) or T cell receptors (TCR) [1].

Effector cells used for adoptive immunotherapy strategies must meet several requirements to ensure a successful outcome of the treatment. First, they must be easily expandable *ex vivo* to get sufficient numbers to achieve relevant clinical responses. Second, they must have a high specificity for the cancer cells to traffic to the tumor site and avoid any damage to healthy tissues. Third, they should be able to proliferate and persist significantly *in vivo*, exerting a sustained and prolonged antitumor response. Importantly, ACT should be safe and well tolerated in patients, generating only mild adverse effects or toxicities.

Several effector cell populations have been developed for ACT purposes, such as Lymphokine-activated killer (LAK) cells [2], Tumor-infiltrating lymphocytes (TILs) [3], CAR- or TCR-transduced T cells [4], NK cells [5], γδ T cells [6], Natural Killer T (NKT) cells [7] and Cytokine-Induced Killer cells (CIK) [8]. This review will focus on CIK cells highlighting differences with other cell populations, as well as the involvement and importance of cytokines in shaping CIK cell features.

1.1. Cytokine-Induced Killer (CIK) cells

CIK cells are a very promising cell population for ACT approaches. They were essentially obtained by the optimization

Abbreviation: ACT, Adoptive cell transfer; ADCC, Antibody-Dependent Cell-mediated Cytotoxicity; CAR, Chimeric antigen receptor; CIK, Cytokine Induced Killer cells; CMV, Cytomegalovirus; EGFR, Human epidermal growth factor 1; GVHD, Graft-versus-Host disease; ICI, Immune Checkpoint Inhibitors; IFN-γ, Interferon-γ; IL-2, Interleukin-2; IL-15, Interleukin-15; LAK, Lymphokine-activated killer cells; mAbs, Monoclonal Antibodies; MHC, Major Histocompatibility Complex; NKG2D, Natural-Killer group 2 member D; NKT, Natural Killer T cells; PBMCs, Peripheral Blood Mononuclear Cells; TCR, T cell receptor; TILs, Tumor-infiltrating lymphocytes.

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of LAK cell expansion protocol, but they differ from these latter cells for some critical aspects.

In the early 1980s, Rosenberg's group described the generation of LAK cells from both murine and human lymphocytes, as a cell population capable of lysing cancer cells after a short-term incubation (from 3 to 5 days) in interleukin-2 (IL-2) [2,9]. These cells were able to lyse a wide array of autologous and allogeneic fresh tumors, and NK-resistant cells [2]. However, LAK cells did not expand efficiently *ex vivo* and therefore alternative culture conditions were investigated, to allow long-term culturing and higher proliferation of effector cells. The use of activation signals such as OKT3, a mitogenic anti-CD3 monoclonal antibody (mAb), in combination with IL-2 led to a significant expansion of effectors with an improved lytic activity [10,11]. Moreover, the incubation of cells with IFN-γ further increased the cytotoxic activity but only if the cytokine was added 24 h before IL-2; IFN-γ priming at the same time or following incubation with OKT3 and IL-2 was much less effective in generating cytotoxic cells [8,12]. Likewise, IL-1 alone had no effect on cytotoxic activity, unless it was combined with IFN-γ and anti-CD3 [8]. Thus, the optimization of the LAK expansion protocol through the definition of a time-sensitive schedule for the addition of IFN-γ, OKT3 and IL-2, led to the obtainment of CIK cells [8,13].

CIK cells are a heterogeneous subset of polyclonal $CD3^+CD56^+$ T cells with phenotypic and functional properties of NK cells. They derive from $CD3^+$ T cell precursors that acquire the expression of CD56 during expansion [14]. CIK cells show a higher proliferation rate than LAK cells, up to 1000 folds, and can be obtained from PBMCs, bone marrow mononuclear cells and umbilical cord blood. After 2 weeks of expansion, the bulk population is mainly composed by $CD3^+CD56^+$ CIK cells and $CD3^+CD56^-$ T cells, and only a small fraction of $CD3^-CD56^+$ NK cells [15,16]. Cytotoxic activity is mainly associated with the $CD3^+CD56^+$ subset, differently from LAK cells in which the major effectors express conventional NK markers ($CD3^-CD56^+$) [2].

Expanded CIK cells also differ from NKT cells, which are mainly defined accordingly to their ability to recognize a relatively monomorphic non-classical class I-like MHC molecule, CD1d, which presents a wide range of lipid antigens from bacterial lipids to mammalian self-lipids [17–19]. Most NKT cells express the same Vα chain (Vα24 in humans and Vα14 in mice), Jα segment (Jα18), single N-region glycine residue, and a limited number of TCRβ chains, namely Vβ8, Vβ7 and Vβ12 in the mouse, and Vβ11 in the humans [20]; thus, NKT cells are also defined invariant NKT cells, or iNKT. CIK cells, instead, express a polyclonal TCR repertoire [21,22].

CIK cells, similarly to LAK cells, do not require antigen-specific stimuli to be activated and proliferate, and exert a potent MHC-unrestricted antitumor activity against both hematological and solid malignancies, but not against normal tissues and hematopoietic precursors [8,23]. CIK cell cytotoxicity is mainly mediated by the engagement of NKG2D and release of perforin and granzyme-containing granules [24]. Preclinical studies and clinical trials have demonstrated the feasibility and the therapeutic efficacy, together with low toxicity, of CIK cells infusion [8,25,26].

These data support CIK cells as a very promising cell population for adoptive immunotherapy. Three crucial properties favorably characterize CIK cells: i) the easy and relatively inexpensive ex vivo expansion; ii) the MHC-unrestricted tumor killing; iii) the reduced alloreactivity across MHC barriers. Each of these aspects will be addressed, outlining the role of the cytokines involved.

2. Cytokines and signals for CIK cell expansion

As described in the previous paragraph, CIK cells grow efficiently *in vitro* relying on a time-sensitive schedule for the addition of IFN-γ, OKT3 and IL-2 to the culture medium.

2.1. Interferon-γ (IFN-γ)

Maximal induction of cytotoxic activity occurs only if the IFN-γ priming precedes by 24 h the mitogenic stimulation with OKT3 and IL-2. Itoh et al. demonstrated that the pre-incubation with IFN-γ induces a differentiation signal that promotes and enhances the IL-2-mediated proliferation [27]. Indeed, IFN-γ itself does not induce proliferation nor cytotoxic activity of killer cells, as demonstrated by culturing in IFN-γ alone, but acts with a synergistic effect enhancing the recruitment and activation of IL-2-responding cells [28]. Upon IFN-γ priming, the IL-2 receptor expression on effector cells is induced, resulting in a higher responsiveness to IL-2 followed by a higher activation [27].

Moreover, IFN-γ activates the monocytes that are present in PBMCs at the beginning of the culture period. Activated monocytes provide two types of signal: first, the contact-dependent signal of CD58 (also called LFA-3), which interacts with the adhesion molecule CD2 expressed on T cells, regulating the responsiveness to IL-12 [29]; second, IL-12 as a soluble factor, which has potent immunomodulatory effects on both T and NK cells, inducing IFN-γ production and proliferation of pre-activated cells [30]. These two signals synergistically promote CIK cell proliferation and increase their cytotoxic activity [31].

2.2. Interleukin-2 (IL-2)

IL-2 is one of the most important cytokines that play extremely important roles in the immune system. It drives CIK cells proliferation and is the only stimulus regularly provided during all culture period, whereas IFN-γ and OKT3 are added only on the first and the second day, respectively.

Besides its potent T cell growth factor activity, IL-2 induces proliferation and cytolytic activity of CIK, NK as well as LAK and TIL cells, and modulates T cell differentiation into Th1 or Th2 cells [32].

IL-2Rα (CD25) is absent or minimally expressed on resting T and NK cells, but is transcriptionally upregulated in T cells stimulated via the TCR or IL-2 [33], or in NK cells stimulated with IL-2 [34]. After T cell stimulation by both IL-2 and IFN-γ, the receptor is rapidly induced and forms high-affinity dimers assembling with the IL-2Rγ, increasing responsiveness to IL-2 [32,35].

IL-2 is crucial for CIK cell *in vitro* expansion, but *in vivo* experiments demonstrated that CIK effector activity is independent of exogenous IL-2 administration [8,14,26]. Notably, one of the major issues in the clinical translation of LAK cells was the relevant toxicity produced by the high doses of IL-2 required to treat patients (approximately 100,000 units per kilogram every 8 h) [36]. CIK cell independence from exogenous IL-2 is an extremely important feature because allows to completely eliminate the IL-2-related side effects in clinical applications.

2.3. Anti-CD3 mAb (OKT3)

OKT3, an anti-human T cell monoclonal antibody, recognizes the CD3 epsilon chain of the human TCR, and stimulates T cell proliferation through TCR cross-linking [37].

The addition of OKT3 to PBMC cultures induces their proliferation through a mechanism dependent on the availability of IL-2, as provides a mitogenic signal subsequently sustained by IL-2. Indeed, the most important transcription targets of OKT3 downstream signaling are IL-2 and its receptor [38].

2.4. Interlerukin-15 (IL-15)

IL-15, together with IL-2, is one of the members of the common γ-chain (γc) family of cytokines [39]. It plays a major role in the survival of NK, NKT and memory $CD8^+$ T cells, and both in the

differentiation and activation of NK cells [40]. Rettinger et al. demonstrated that the stimulation with IL-15 induces a significant enhancement of CIK cell (CIK_{IL-15}) cytotoxicity against acute myeloid (AML) and lymphoblastic leukemia/lymphoma (ALL) cell lines, as well as against primary human AML and defined ALL leukemia cells, compared to conventional IL-2-activated CIK cells (CIK_{IL-2}) [41]. The IL-15-modified protocol consists in the expansion of CIK cells with the standard protocol (IFN-γ, OKT3, IL-2) for the first 3 days, followed by the addition of IL-15 from day 4 of culture. The percentages of $CD3^+CD56^-$ T cells, $CD3^-CD56^+$ NK cells and $CD3^+CD56^+$ CIK cells among CIK_{IL-15} did not significantly differ from CIK_{IL-2}. However, the percentage of $CD3^+CD8^+$ $CD25^+$ activated T cells was significantly increased in CIK_{IL-15} cultures. No differences in the proliferative capacity between CIK_{IL-15} and CIK_{IL-2} were observed over a culture period of 21 days. Nevertheless, the crucial role for the NKG2D receptor in triggering cytotoxicity was confirmed for CIK_{IL-15}, as well as the low alloreactive potential against allogeneic PBMC and fibroblasts [41]. With subsequent *in vivo* experiments, the same group demonstrated that CIK_{IL-15} retain strong anti-leukemia activity *in vivo*, and are able to traffic and survive in AML and sarcoma mouse models for a prolonged time with minimal GVHD, as demonstrated by histological analyses of gut, liver, and spleen of treated mice [42].

3. CIK cell MHC-unrestricted tumor killing activity

One of the most important and distinctive features of CIK cells is their MHC-unrestricted antitumor activity against a broad range of tumor histotypes. This activity is mainly associated with the $CD3^+CD56^+$ subset, does not require prior antigen exposure or priming, and is not exerted against normal tissues and hematopoietic precursors [14,21,23].

Functional assays using blocking antibodies against CD3, CD8, CD56, TCRα/β, and MHC class I and II molecules failed to inhibit cytotoxicity, demonstrating that CIK cells recognize target cells by TCR- and MHC-independent mechanisms; moreover, the cell-to-cell contact is strongly required for cytolysis, as proved by the significant inhibition of cytotoxicity induced by blocking CD11 and ICAM-1 [12,43,44].

The molecule that plays the most important role in tumor recognition by CIK cells is NKG2D, a member of the c-type lectin-activating receptor family that is normally expressed on all NK cells, and interacts with receptors almost exclusively expressed in malignant tissues [45,46]. NKG2D is upregulated by IFN-γ, IL-2 and TCR-crosslinking with OKT3 antibody; thus, it appears highly expressed in expanded CIK cells while not being restricted to the $CD3^+CD56^+$ subpopulation only. The main known ligands for NKG2D are stress-inducible molecules, such as the MHC class I-related molecules A and B (MIC A/B) and members of the UL16-binding protein family (ULBP1-4) [23,24]. The receptor engagement triggers IL-2-activated NK cells, and induces calcium flux, cytokine release and cytotoxicity [47]. Studies with antibodies blocking the NKG2D receptor, siRNA experiments and redirected cytolysis indicated that the majority of the MHC-unrestricted cytotoxicity of CIK cells is exerted through the NKG2D interaction rather than the TCR engagement [23,24,48].

While NKG2D mediates the interaction between CIK cells and tumor targets, the final cytolytic effect is perforin- and granzyme-mediated; in fact, CIK cells generated from perforin-knockout mice completely lack their antitumor ability [49].

Even though most of the cytotoxicity is mediated by NKG2D, CIK cells can also retain TCR-mediated lytic activity, showing a "dual-functional capability" [43]. In this regard, Pievani et al. induced CIK cells from the PBMCs of CMV-seropositive healthy donors, obtaining a remarkable increase of CMV-specific $CD3^+CD56^+$ CIK cells. The same cell population could mediate antitumor activity based on both TCR-dependent (against autologous cells loaded with CMV peptides) and TCR-independent cytotoxicity (NK-like, MHC-unrestricted cytotoxicity against tumor cell lines and freshly isolated leukemic blast). Notably, an anti-NKG2D blocking mAb affected the non-MHC-restricted cytotoxicity but had no effect on TCR-dependent killing of CMV-pulsed autologous blasts, thus demonstrating a role in the NK-like cytotoxicity only. In contrast, blocking the binding between effectors and target cells by addition of anti-LFA-1 mAb induced a strong inhibition of both lytic pathways [43].

Recently, our group reported the remarkable observation that CIK cells present a donor-dependent expression of CD16, and that the concurrent administration of therapeutic mAbs, such as trastuzumab or cetuximab, leads to a significant improvement in their antitumor activity, triggering a potent antibody-dependent cell-mediated cytotoxicity (ADCC) both *in vitro* and *in vivo*. Interestingly, ADCC activity is accountable to $CD3^+CD56^+CD16^+$ CIK cell fraction, as the removal of both NK cells and $TCR\gamma/\delta^+$ CIK cells did not affect the enhancement of cytotoxicity induced by mAbs [50]. This mAb-mediated increase of CIK cell antitumor potential could allow reducing the total cell dose and the number of infusions required for treatment, with positive implications for patients with limited *ex vivo* expansion rates of CIK cells [51].

The MHC-unrestricted cytotoxicity of CIK cells has the potential to bypass HLA restrictions in the therapeutic settings, and thus offers interesting perspectives to widen the number of patients that could be treated with a CIK cell-based therapy. Moreover, such MHC-unrestricted tumor recognition might help to overcome important immunological tumor escape mechanisms, like the downregulation of MHC molecules and the improper presentation of tumor associated antigens.

4. Role of CIK cells in GVDH

Graft-versus-host disease (GVHD) is the most frequent and severe complication associated with the adoptive infusion of allogeneic lymphocytes. Since from the early *in vitro* studies, CIK cells appeared to be endowed with a reduced alloreactive potential compared with conventional T cells, even when challenged across MHC-barriers. This observation was subsequently confirmed by preclinical studies in animal models that helped to highlight crucial mechanisms responsible for the reduced GVHD.

To explore CIK cell GVHD potential *in vivo*, experiments were performed in both syngeneic and allogeneic settings. In the allogeneic setting, the injection of up to 20×10^6 CIK cells did not result in clinically significant GVHD, whereas as few as 2.5×10^6 splenocytes induced acute lethal GVHD [26]. This could be explained by the relevant endogenous production of IFN-γ by CIK cells [49], which is known to be protective against GVHD [52]. This hypothesis was confirmed by generating CIK cells from IFN-γ knockout mice, which rapidly induced lethal GVHD when infused across MHC-barriers with a kinetics similar to unmanipulated splenocytes. On the other hand, CIK cells expanded from IL-2, Fas, FasL, and perforin knockout animals behaved as the wild-type cells, inducing just little to none GVHD across major histocompatibility barriers. Interestingly, the IFN-γ production is required by the donor cells and not by the recipient animals, since wild-type CIK cells injected into IFN-γ knockout animals did not cause GVHD [49].

Sangiolo et al. observed that in bulk-expanded CIK cell populations the $CD3^+CD56^-$ and the $CD3^+CD56^+$ subsets can be distinguished according to their phenotype and function [23]. Indeed, expanded CIK cells are alloreactive across major HLA barriers only when tested as a bulk population, similar to fresh lymphocytes. If tested separately, it was clear how the majority of

the observed alloreactivity was due to the $CD3^+CD56^-$ subset, whereas $CD3^+CD56^+$ cells showed only minimal alloreactive capacity while maintaining their antitumor activity [23]. The $CD3^+CD56^-$ subpopulation retains its alloreactive potential, and is responsible for the GVHD reactions. Thus, for clinical applications, the depletion of the alloreactive CIK cell subpopulation would not affect the tumor killing capacity, and could help in extending this approach across major HLA barriers, further reducing the risk of severe GVHD and preserving the anticancer effect [23].

An additional explanation for the reduced alloreactivity comes from the observation that, upon allogeneic stimulation, the upregulation of homing molecules and chemokine receptors, which are necessary to enter the inflamed and GVHD target organs, was much slower and its peak lever lower in CIK cells compared to conventional T lymphocytes [26]. Both allogeneic CIK cells and naïve splenocytes homed to and proliferated in GVHD target organs with a similar pattern, homing to spleen and lymph nodes with an expansion peak within the first week. However, CIK cells division rate *in vivo* was much slower than that of naïve T cells, thus avoiding the death in all recipient mice due to acute GVHD. Moreover, CIK cells are more susceptible to apoptosis, as demonstrated by annexin V and PI staining of donor-derived CIK cells [26].

Clinical trials confirmed the feasibility of this approach and the reduced propensity of these cells to cause GVHD. Mild GVHD occurred in a very small portion of patients treated with allogeneic CIK cells, and in all cases, symptoms were responsive to corticosteroid treatment. Besides GVHD, the most common side effects of CIK cell therapy are mostly mild, with fever, headache, fatigue, rashes, nausea and vomiting, which are easily controllable and well tolerated [25].

5. CIK cells and immune checkpoint inhibitors

Tumors can promote an immunosuppressive activity, leading to a major survival, proliferation and metastatization. An important challenge today is to understand how the different components of the tumor microenvironment inhibit the immune response [53], and how these could be blocked. Recently, the use of immune checkpoint inhibitors (ICI) showed highly satisfactory clinical results in patients with advanced cancers and treated with ACT [54].

In this regard, just a few studies have been reported that employed CIK cells in combination with monoclonal antibodies against immune checkpoints. The first clinical evidence was highlighted in 2015, when one patient with an advanced squamous non-small cell lung cancer (NSCLC) was treated with 13×10^9 CIK cells in combination with 2 mg/kg of Pembrolizumab (anti-PD-1 mAb) every 3 weeks. A rapid tumor regression was already noticed after 2 cycles of Pembrolizumab and one of CIK cells, while after 5 cycles of antibody and 3 of immune effectors the PET-CT scanning evidenced the reduction of tumor burden and radiotracer uptake. Notably, this high effective therapy was well tolerated [55]. In addition, a recent study confirmed that the combination of CIK cell infusions with a prior treatment with ICI can improve the therapeutic efficacy in NSCLC patients. This observation argues against the consideration that CIK cells isolated from NSCLC patients could be exhausted before the infusion, due to the dynamic profile expression of different immune checkpoint receptors during the cell culture period. After 2 weeks of culture, CIK cells maintained a high expression of TIM-3, LAG-3, PD-L1 and CEACAM-1, but low expression of PD-1, TIGIT, BTLA and CTLA-4 [56,57]. Hence, it would be important to select the best-fitting ICI to infuse in patients treated with CIK cells in ACT, to prevent immunosuppression activities, and to understand whether this strategy could be applied in all tumor histotypes or if it depends on the type of the tumor treated.

A very recent work evaluated the expression (which was comparable between healthy donors and patients with hematological malignancies) of the inhibitory receptors BTLA, CTLA-4, CD200R, KIR2DL1/2/3, LAG-3, PD-1 and TIM-3 on CIK cells at day 21–23 of culture. This work underlined the high expression of TIM-3 over the other receptors, and showed that TIM-3 blockade led to the highest increase in cytotoxicity; conversely, CTLA-4 blockade did not potentiate the lytic activity, as the receptor is present at low levels. Likewise, the combination of the two blocking antibodies did not further enhance cytotoxicity. However, CIK cells disclose a much higher cytotoxicity than the unactivated T and NK cells, supporting the concept that CIK cells may be a promising strategy to use with ICI to achieve a superior clinical outcome [56].

A simultaneous PD-1/PD-L1 pathway blockade along with CIK cell infusion was proved as a potential novel immunotherapy approach for gastric and colorectal cancer in preclinical models, where the combined therapy induced a delay in tumor growth and a survival advantage respect to the untreated mice. In this study, the enhancement of CIK cell cytotoxicity was demonstrated indirectly by a large release of associated immune–promoting molecules, such as IFN-γ, the expression of CD107a, and the increase of NKG2D receptor levels due to the PD-1/PD-L1 pathway blockade [58].

Interestingly, in a retrospective study on hepatocellular carcinoma (HCC), patients with $\geq$5% PD-L1 expression had a better overall survival and recurrence-free survival than patients with 1–5% or $<$1% PD-L1 expression if treated with CIK cell immunotherapy, confirming that the PD-L1 expression in HCC could be used as a biomarker for predicting survival benefits from CIK cell immunotherapy. The high expression of PD-L1 on tumor cells indicates a mechanism potentially capable of interfering with the MHC-restricted antitumor immune responses, and CIK cells might be a good choice to induce a more potent therapeutic efficacy [59].

Up to date, there are only two ongoing clinical trials that study the role of CIK cells in combination with ICI. The first is a Phase II study, in which patients with different tumors (HCC, renal cell carcinoma, bladder, colorectal, NSCLC and breast cancer) will receive 4 cycles of treatment at 2-week intervals, or until disease progression occur, of autologous dendritic cells and CIK cells (D-CIK) in combination with anti-PD-1 mAb (NCT02886897) (https://clinicaltrials.gov/ct2/show/NCT02886897?term=cik&rank=12).

The second study investigates the combination of CIK cell infusions with ipilimumab (anti-CTLA-4 mAb) in patients with stage I metastatic melanoma. In this Phase II study, patients will receive 4 injections of ipilimumab at 3-week intervals, with or without CIK cells. This therapy should result in tumor stabilization or shrinkage, significant prolongation of progression-free, disease-free or overall survival, compared to the use of ipilimumab alone (NCT02498756) (https://clinicaltrials.gov/ct2/show/NCT02498756?term=NCT02498756&rank=1).

6. Redirecting CIK cells with chimeric antigen receptors

The chimeric antigen receptor T cell (CAR-T) therapy is a newly developed ACT antitumor treatment. CAR-T cells can specifically localize and eliminate cancer cells by interacting with the tumor-associated antigens (TAAs) expressed on tumor cell surface. Immunotherapy using CAR-T cells is now being investigated in several clinical trials both in hematologic diseases and solid tumors, showing promising outcomes [60].

CIK cells raised interest for use in cellular antitumor therapy due to their capability to recognize and destroy autologous tumor cells in an HLA-independent fashion. Nonetheless, this antitumor efficacy could be improved by redirection with CAR molecules, which recognize the cancer cells and then trigger CIK cell

activation. Nevertheless, there are limited data regarding CAR-modified CIK (CAR-CIK) cells.

An important study by Hombach et al. investigated the efficacy of second and third generation carcinoembryonic antigen (CEA)-specific CAR molecules in inducing the best antitumor activity in CIK cells against colon carcinoma. CAR-redirected CIK cells benefited from a CD28 co-stimulation, whereas the addition of further co-stimulation by OX40, as found in third generation CAR (CD28-ζ-OX40), induced less antitumor efficacy due to increased activation-induced cell death (AICD) [61]. In contrast, a very recent study from Zuo et al. has shown better results using a third generation folate receptor-specific CAR (CAR-FRα), with an increased CIK cell proliferation, IL-2 release, and enhancement of cytotoxicity *in vitro*, and a long-term suppression of tumor and improved results *in vivo*, as compared to a first-generation CAR that induced an increase of IFN-γ but a short-term suppression of tumor [62]. Based on our unpublished results, these discordant results could be explained by the inherent different affinity/avidity of CAR molecules for their receptors. Intrinsically low-affinity CAR-FRα in ovarian cancer might require a more effective co-stimulation to trigger a strong response, as compared to the CAR-CEA in the colon carcinoma model that might have better affinity/avidity for its ligand, and thus does not need further co-stimulation that on the contrary could foster an excessive signaling and activation, leading the effector cells to AICD.

Promising results using CAR-CIK cells have been reported against acute lymphoblastic leukemia (ALL), showing complete and durable molecular remissions of established primary pre-B-ALL as compared to the treatment with unmodified CIK cells [63]. CAR-CIK cells bearing anti-CD33 and anti-CD123 CAR [64] and recognizing acute myeloid leukemia disclosed an increased proliferative activity, with the release of high levels of immunostimulatory cytokines [65,66].

CAR-CIK cell therapy represents a promising option also for the treatment of solid tumors. Ren. et al. genetically modified CIK cells to express a CAR molecule directed against the Epidermal growth factor receptor (EGFR), a cell-surface molecule overexpressed in a variety of solid tumors including NSCLC, breast, head and neck, esophageal, gastric, colorectal, prostate, bladder, renal, pancreatic, and ovarian cancers [67]. They reported an increase of the cytotoxicity and IL-2 and IFNγ release *in vitro,* and a decrease of tumor growth *in vivo*, with an improvement of survival and CAR-CIK cell persistence in mouse peripheral blood [68].

7. Concluding remarks

CIK cells are a very attractive tool for adoptive immunotherapy approaches against hematological and solid tumors. They can be easily expanded using a straightforward and inexpensive expansion protocol, and are safe as they only require GMP-grade cytokines to obtain very high amounts of cytotoxic cells. CIK cells have shown promising results in clinical trials, exhibiting a good profile of safety and tolerability due to the almost complete lack of GVHD activity, even in allogeneic settings. Moreover, the independence from the administration of exogenous IL-2 avoids the cytokine-related adverse effects in patients.

Overall, the evidence obtained from a growing body of literature highlight the complexity of the mechanisms underlining CIK cell-mediated cytotoxicity, and the plethora of opportunities that CIK cells offer when used in ACT approaches. CIK cells can be exploited for clinical use not only for their intrinsic MHC-independent antitumor properties, but also for the possibility to be redirected against specific tumor antigens by already available molecular tools, such CAR molecules. More interestingly, they can acquire antigen-specificity by the simple combination with clinical-grade mAb, bypassing the expensive and technically challenging steps of genetic manipulation. Importantly, the simple change of the mAb could broad the approach to other different cancer types. Furthermore, the combination of ICI therapy with CIK cell ACT may yield a superior clinical outcome compared to the many currently ongoing clinical trials employing effector cell monotherapy or chemotherapy combinations.

Thus, a very fascinating story started with LAK cells has evolved and improved, ending up with the optimization of an effector cell population that holds great promises for cancer therapy.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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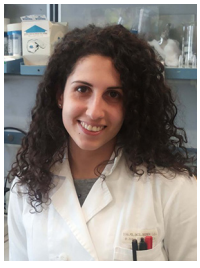

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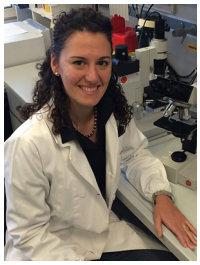

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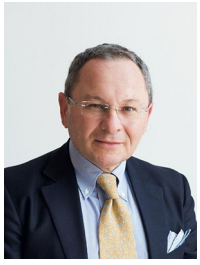

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