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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