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CD4+ T-HELPERS IN TCR-DEPENDENT TUMOR IMMUNOSURVEILLANCE AND T-CELL BASED ADOPTIVE TRANSFER IMMUNOTHERAPY: ARE THEY REALLY THAT HELPFUL?

ABSTRACT

In this brief review the advances and hurdles of the modern-day ACT (adoptive cell transfer) immunotherapy of cancer are discussed, with the focus on the positive or negative role of $CD4^+$ T helper lymphocytes as one of major constituents of oncologic patient-administered CIK (cytokine-induced killers) lymphocyte culture. The beneficial role of $CD4^+$ T helpers in adoptively-transferred lymphocyte culture is considered, questioned and being put under doubt. "Infectious tolerance" and tumor "immune avoidance" phenomena are described, emphasizing on their dramatic implications for cancer ACT therapy. The ways to circumvent apparent undesired effects of $CD4^+$ T helpers elevated presence in CIK bulk mass are discussed, such as complete removal of CD4 – positive cells, along with a less radical measure, which is depletion of $CD4^+CD25^+FoxP3^+$ T regulatory lymphocytes from bulk CIK culture.

KEYWORDS: *cancer immunotherapy; adoptive cell transfer; cytokine-induced killers; CD4+ T-regulatory lymphocytes*

ACT (adoptive cell transfer) is a branch in immune-based therapy of cancer, which is dynamically developing during the last 20 years. Basically, it is ex vivo culture and expansion (up to 1000-fold or even more) of oncologic patient's autologous (or in some cases allogeneic) lymphocytes, with subsequent infusion back to patient's bloodstream. Usually this treatment is conducted in combination with (largely after) more traditional treatments, such as surgery, radio- and especially chemotherapy; and is intended to eliminate residual tumor burden, which is left after these courses [1-5]. ACT therapy is based on the fact, that immune system can recognize and extinguish malignantly transformed cells; although this ability is attenuated due to number of reasons (so called "tumor immune escape / avoidance" phenomenon) [6-11], which will partly be discussed in this brief review.

Firstly known as LAK (lymphokine-activated killers), nowadays these cells are mostly referred as CIK (cytokine-induced killers). For clinical application, they are largely obtained and grown from 3 main sources: either from surgically-derived tumor biopsy (in this case they are called TIL: tumor-infiltrating lymphocytes); either from resected tumor-draining lymph node ("sentinel lymph node lymphocytes"); either from patient's blood or leukopheresis product, i.e. from PBMC (peripheral blood mononuclear cells) [1, 2, 12-26]. Basic process schedule normally includes isolation of lymphocytes from the whole white blood cell mass via density-gradient centrifugation; followed by stimulation using either tumor-antigen pulsed dendritic cells, either with a combination of IFN-γ + anti-CD3 mAb (often additional mAbs, commonly anti-CD28); followed by culture and expansion in growth medium with added cytokines (usually IL-2 and other) [1, 3-5, 11-13, 22, 24-25, 27-28].

During the recent few years, advanced approaches, which involve the transfection of CIKs with genetically-engineered tumor-antigen-specific natural or chimeric TCRs, are also rapidly emerging and ongoing multiple successful clinical trials [3-5, 11-12, 18, 25]. Also, ACT can be combined with such treatments as administration of "immune checkpoint" blocking monoclonal antibodies [9], bispecific antibodies [2-4] and other approaches, which have been shown to increase ACT efficacy. Numerous ACT strategies which use CIK have been already approved for clinical use in many countries (e.g. USA, China, countries of European Union, etc.), and significant therapeutic success has been achieved in many cases. Depending on clinical protocol and on properties of specific tumor (for instance, melanomas [11, 16, 17, 26] and lung cancers [11] are often highly immunogenic, thus CIK therapy works particularly well against them), range of ~30% to ~80% of partial and even complete clinical response rates has been achieved [3, 5, 16, 18, 29- 30].

CONSTITUENTS OF CIK: TH1, CTL, NK, NKT

CIKs are not a homogenous cell population. It's a complex mixture of different lymphocyte subtypes, main of which are $CD3^+CD4^+$ T helpers, $CD3^+CD8^+$ cytotoxic lymphocytes (CTL, previously referred as T killers), $CD16^+CD56^+$ natural killers (NK), and a small proportion of cells which share characteristics of both NK and T cells – $CD3^+CD56^+$ NKT [1- 2, 13-14, 16-18, 26-27, 31-32].

Previously, at early stages of development of ACT cancer immunotherapy, it was considered, that $CD4^+$ T helpers play the major role in antitumor response within CIK/TIL mixture. For instance Steven Rosenberg, one of big gurus in immunotherapy, has shared this view for many years [18, 29, 30].

However, Rosenberg himself, along with another researchers, acknowledges, that only a limited clinical success of genetically-unmodified T-helper-focused adoptive transfer therapy has been achieved so far [7, 25-26].

So it looks like, that benefit of $CD4^+$ T helpers –based approach for ACT is a big issue.

PROBLEMS ASSOCIATED WITH ALL $CD3^+TCR^+$ T CELLS

First, extensively-cultured, antigen-exposure exhausted, highly-differentiated T cell lines are prone to senescence and apoptosis, and therefore have a short survival time after infusion to a patient's body [17-18, 25-26].

Second, TCR high specificity leads to a fact that only a very limited percentage of T cell clones are reactive to specific tumor antigens. This issue can be partly overcome (apart from genetic modification) by pulsing of bulk CIK culture with a tumor lysate or particular tumor marker antigen, followed by selection of useful, i.e. Ag-reactive clones with the help of cytotoxicity- or cytokine-secretion assays and choosing them for further culture and expansion [30].

Third, majority of tumor antigens (apart from oncogenic-virus Ags and neo-Ags which bear a mutation) are self-Ags; although expressed either ectopically, either at abnormally-high levels, either at a wrong developmental stage. It makes them weakly-immunogenic [11, 33], and immune response to them is tightly controlled by natural and induced T regulatory lymphocytes, which will be discussed in the section below.

Fourth, tumor-Ag-specific T cells are highly prone to be "turned off" by ligands to so-called "immune-checkpoint" receptors [9], or incited to undergo apoptosis by proapoptotic ligands (e.g. FasL etc.), which have been shown to be highly expressed on many tumors [7].

$CD4^+$ T HELPERS: EVEN MORE PROBLEMS

And moreover, in addition to the above-listed limitations of T-cell-focused immunotherapy, $CD4^+$ T-helpers seem to be the "trickiest" subset of effector T lymphocytes. $CD4^+$ T helpers take part in amplifying immune response via secreting immune-boosting cytokines such as IL-2, IFN-γ etc.; and by "triggering on" B cells and $CD8^+$ cytotoxic lymphocytes [2, 30].

However, there are reports, stating that T-helpers contribute only to a very limited extent to antitumor action of CIK [16, 32]. The main point of T helpers is regulation of immune response, boosting it or quenching it when necessary or, in pathology, when being disregulated (e.g. in cancer) – when unnecessary. By the way, it has been stated previously, that Th1 cells are more susceptible to suppression and/or anergy than $CD8^+$ cells [34].

Th1/Th2 differentiation also may be involved: Th2 subset has been shown to have immune-modulatory and even cell-immunity-suppressive properties by many researchers [6, 10, 35-38]. Quite remarkably, only Th1 subset is immune-response-promoting subpopulation of $CD4^+$ cells (and even then – only under certain conditions); whereas above-mentioned Th2 and especially $CD4^+CD25^+FoxP3^+$ T regulatory lymphocytes (Tregs) [2-3, 5, 8-10, 13-14, 16-18, 21, 23-24, 32, 34, 36-49] and Th3 [37-38, 40-43] are immune-response quenching subsets.

Direct citation from an excellent review on natural T regulatory cells clearly says: "It is clear that all forms of $CD4^+$ T cells can acquire the ability to make immunoregulatory cytokines such as interleukin 10, transforming growth factor-β and interleukin 4. Such cells were formerly called T helper type 2 and T helper type 3 and were discussed in terms of immune deviation or class regulation" [38].

Conclusions of Shevach E. [37] are also consistent with this – according to the author of this review about Tregs mechanism of action, Th1 cells under certain conditions can definitely exert immunosuppressive properties via secreting immunohibitory cytokines, such as IL-10 and TGF-β [37]. The reasons and mechanisms how and why $CD4^+$ T helpers can acquire immunosuppressive properties, are discussed in the section below.

TUMOR IMMUNE ESCAPE: INDUCTION AND RECRUITMENT OF TREGS AS ONE OF ITS MAJOR MECHANISMS. INFECTIOUS TOLERANCE

The results of elegant studies [44-45], and especially [46], at which "infectious tolerance" phenomenon has been discovered and investigated, have important implications for CIK cancer immunotherapy, especially for T-helper based approach, which many therapeutically-engaged companies focus on. Here we see, that presence of small population of $CD4^+CD25^+FoxP3^+$ Tregs in proliferated and adoptively transferred CIK bulk culture, under certain conditions can lead (in cascade-like, amplifying fashion, due to "infectious tolerance" phenomenon) to generation from "good" $CD4^+CD25^-FoxP3-$ T helpers of large quantity of regulatory lymphocytes with immune-suppressive properties, which can persist *in vivo*, i.e. in patient's body; and at least, hinder antitumor function of transferred CIK, or even lead to more deep immune-suppression [44-46].

Few direct citations from two good review articles will be appropriate here:

"Constitutive presentation of self-antigens by immature dendritic cells; high levels of transforming growth factor-β and prostaglandin E2 (derived in part from elevated COX-2 expression) in the tumor microenvironment may also enhance the conversion of FoxP3-negative $CD4^+$ T-cell effectors to FoxP3-positive regulatory T cells. In transplantable tumor models, the administration of antibodies to CD4 or CD25, which effectively antagonize regulatory T-cell function, established a critical role for regulatory T cell–mediated immune suppression at both early and late stages of disease, as these manipulations evoked impressive tumor regressions and protection against subsequent tumor challenges" [8].

"Although there is commonly significant infiltration of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocyte (CTL) cells at the tumor site, tumor cells can use immunosuppressive strategies to induce $CD4^+$ and $CD8^+$ T-cell anergy and create a tolerant tumor microenvironment. Antigen-presenting cells play a crucial role in tolerizing tumor antigen-specific $CD4^+$ and $CD8^+$ T cells. Tumors may subvert tumor immunity by promoting the expansion, recruitment, and activation of regulatory T (Treg) cells. $CD4^+$ Treg subsets include naturally occurring $CD4^+CD25^+$ Treg cells as well as peripherally induced $CD4^+$ Treg cells" [10].

Antigen-presentation to naïve $CD4^+$ T helpers by immature and certain tissue-specific DC (dendritic cells; especially by plasmacytoid DC and immature myeloid DC) [8, 37, 42, 46-47, 51], and/or Ag-presentation at suboptimal conditions in the presence of immune-inhibitory cytokines TGF-β and IL-10 [8, 44, 49-50] also leads to conversion of $CD4^+$ T effectors to $CD4^+CD25^+FoxP3^+$ Tregs. Many tumors either directly secrete TGF-β and IL-10, along with other immune-suppressive molecules, either incite non-transformed cells (such as TIM/TAM – tumor-infiltrating/associated macrophages) and myeloid-derived suppressor cells in tumor microenvironment to do so [9, 18, 32-34, 49].

By the way, targeting of T regulatory lymphocytes in cancer patient's body (either through depletion, either through inactivation, either through disrupting the mechanisms of Tregs recruitment by tumor) is one of promising directions in cancer immunotherapy [33, 47, 52], which can have huge synergistic positive effect when combined with CIK ACT approach.

Moreover, some FoxP3-negative suppressor T-lymphocyte populations Tr1 and Th3 can be induced from $CD4^+CD25^-$ T effectors

under certain conditions; e.g. such as antigen-stimulation of naïve $CD4^+$ T helpers in presence of TGF-β/IL-10 [41, 43, 51], which, as has been mentioned above, are often present in tumor microenvironment.

Also worth attention, that only $CD4^+$ T helpers, but not $CD8^+$ CTL, transiently express the crucial Tregs transcription factor FoxP3 upon activation – induction of Foxp3 is cell-type specific and was not found in $CD8^+$ T cells [10]

This is also the case with other immune-inhibitory molecules (e.g. CTLA-4, CD25, OX40/CD137), which are constitutely expressed on Tregs, and transiently – on recently activated T effector cells, largely on $CD4^+$ T helpers [10, 37, 39-40, 47, 49-50].

CLINICAL RESULTS

High level of $CD4^+$ cells (up to 85%) in transfected bulk lymphocyte culture might be the cause of absence of any therapeutic success in several clinical trials with the use of anti-cancer chimeric antigen receptor (CAR) transduced T cells; whereas in those trials where transfected cultures contained relatively low (~45%) $CD4^+$ T cells ratio, significant therapeutic success has been achieved [3].

In a number of highly-successful clinical trials with CAR- or natural antigen-specific αβ-TCR- transfected T cells, the majority of tumor-infiltrating, malignancy-attacking players (as the analysis of post-treatment biopsies has indicated) were not $CD4^+$, but $CD8^+$ αβ-TCR/CAR transfected cells [4-5, 11].

POSSIBLE SOLUTIONS

Taken together all the above-said, there's no surprise, that some therapeutic centers, which perform ACT, nowadays deplete $CD4^+$ cells (mostly using MACS (magnetic-associated cell sorting)) from full-grown clinical grade CIK cultures, in order to obtain $CD8^+$ CTL-enriched CIK population [12].

The less radical approach would be the depletion or inactivation of $CD4^+FoxP3^+(CD127^+)CD25^+$ Tregs from expanded clinical bulk CIK cultures, which is most practically achieved by using MACS – a procedure which has been proposed by number of researchers [2, 14]. Designing of culture protocols that disfavor Tregs proliferation (e.g., addition of IL-7 and IL-15 to cytokine mixture) can also be an option [13]. These measures would prevent cascade-like generation of large quantity of $CD4^+$ iTregs from $CD4^+$ T helpers caused by "infectious tolerance" *in vitro*; but it would probably fail to do so *in vivo*, i.e within a patient's body, in a tumor immunosuppressive microenvironment.

Another direct citation will be to place here:

"Tumour-induced expansion of regulatory T (Treg) cells is an obstacle to successful cancer immunotherapy. In theory, the functional inactivation of Treg cells will maintain them at high numbers in tumours and avoid their replenishment from the peripheral lymphocyte pool, which has the capacity to further suppress the effector lymphocyte anti-tumour response" [49].

SUMMARY

Summarizing all the above said, the mass of experimental and clinical evidence suggests, that $CD4^+$ T helpers are highly versatile, unsteady population of lymphocytes, which is due to their main function – to regulate immune response, by boosting or inhibiting it when necessary via becoming iTregs. Under certain conditions, such as oncological diseases, with their well-characterized ability to suppress immune response [6-11, 32, 49], $CD4^+$ T helpers may play harmful, rather than beneficial role. This consideration should be carefully weighed when designing immunotherapeutic strategies for treatment of cancer. MACS depletion of all $CD4^+$ cells, both T helpers and Tregs; or a less radical measure – removal of $CD4^+CD25^+FoxP3^+$ T regulatory lymphocytes can be proposed, as it may improve the therapeutic outcome of malignant diseases treatment via CIK ACT.

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