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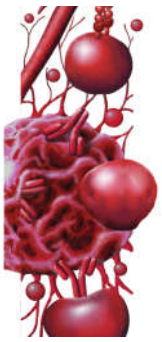

Benefits of a continuous therapy for cancer patients with a novel adoptive cell therapy by cascade priming (CAPRI)

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A growing body of evidence shows that immune cells are pivotal in the fight against cancer. First, association studies have identified immune-protective immune response genes against cancer. Second, the presence of immune cells in the respective malignant tumor correlated with a better prognosis for the patients. Third, adoptive cell therapy (ACT) showed, in recent reports, an efficient reduction or even cure for malignant tumors. The focus of this review is a novel *in vitro* ACT technique, using the patient's cascade-primed immune cells. The cascade-priming procedure stimulates APCs from the peripheral blood. Stimulated APCs digest and present tumor material better and differentiate naive cytotoxic T-lymphocyte effector cells against the patient's cancer. The principle and the impressive results of the cascade-primed immune cell treatment in patient case series is compared with the ACT concepts of lymphokine-activated killer, macrophage-activated killer, macrophage-activated killer-dendritic cell, cytokine-induced killer and tumor-infiltrating lymphocyte methods.

Keywords: adoptive cell therapy • cancer • CAPRI • monocyte • T lymphocyte

The cascade-primed immune (CAPRI) cell therapy is a novel adoptive cell therapy (ACT) for the treatment of cancer. In ACT, the patient's immune cells are prepared outside the body to attack cancer cells and then injected back into the patient. The CAPRI cell procedure makes use of the patient's cancer information that is stored in monocytes to differentiate naive T lymphocytes into cytotoxic effector cells. Immune cells prepared with the CAPRI cell procedure appear to be suitable for most if not all cancer types and can be started within a week after isolation of the blood sample. The CAPRI cell procedure does not require gene manipulations or the identification of immunogenic cancer peptides and is well tolerated by the patients. It can be administered in an adjuvant fashion with chemotherapy or radiation. In Europe, the CAPRI cell therapy is performed only in an immunotherapy center in Munich. In China, seven universities have been using the CAPRI cell method since 2009. As ACT still meets skepticism, a short retrospective is presented in the next section on the first observations that initiated the development of ACT and the modalities of the CAPRI cell therapy.

Immune cell therapy of cancer was considered futile for a century

Nearly a 100 years ago, Peter Gorer observed that the transplantation of a malignant tumor from one mouse to another was usually followed by the rejection of the tumor [1]. However, the malignant tumor grew well and was not rejected if transplanted to a syngeneic host. This was similarly and inadvertently demonstrated in man. A transfusion of leukocytes from a mother with chronic myeloid leukemia to a daughter, suffering from leukocytopenia, caused chronic myeloid leukemia in the daughter. Sufficient matching of histocompatibility antigens between daughter and mother prevented the rejection of the 'transplanted' leukemia cells. This and other observations shaped the opinion that immune cells cannot react against cancer cells because they do not 'really' differ from the other cells of the

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patient. The minute difference between cancer cells and other cells of the body was described as retrodifferentiation into the embryonic stage of the cell. Mutations that transformed a normal cell into a malignant one were considered to be an unhappy event among the trillions of cell divisions occurring daily in the body. Environmental factors seemed to be only occasionally involved in mutational events. Infections with microbes as the essential driving force in the development of cancer did not become the main focus of cancer research despite the discovery of Rous sarcoma virus RNA in the sarcoma of the chicken at the beginning of the 20th century [2]. Homologs of viral oncogene sequences were shown to be present in the human genome, yet the identified oncogenes seemed to regulate cellular functions like the (normal) proto-oncogenes [3]. It was therefore suggested, that the mutation of a single (normal) proto-oncogene into an oncogene is not sufficient for the transformation of a cell. Varmus, who received the Nobel prize together with Bishop for their groundbreaking work on oncogenes, estimated therefore, that five to ten oncogene mutations are required to transform a cell into a cancer cell [4]. The existing mutations plus the numerical increase of mutations caused by environmental carcinogenic factors appeared to Varmus as an insurmountable hindrance for future therapies. It was not known at that time, that proteins of oncogenes can be recognized by immune cells just as viral proteins [5]. The discovery of lymphocytes, which infiltrate malignant tumors (tumor-infiltrating lymphocytes [TILs]) seemed to support the notion that the immune system cannot efficiently respond against autologous cancer cells. First experiments showed that TILs recognize but do not lyse cancer cells. However, as discussed later, TILs from melanomas can lyse autologous cancer cells if cultured *in vitro* in culture medium supplied with stimulating cytokines and allogeneic feeder cells [6,7]. Thus, immune cells can not only distinguish between the normal cell (the melanocyte) and the malignant cell (the melanoma), they can also lyse the malignant cell. This fundamental condition for any ACT in the clinical setting achieves a novel level of performance with the CAPRI cell therapy. First, the CAPRI method does not depend on tumor tissue for the isolation of lymphocytes because *in vitro*-stimulated monocytes from the peripheral blood express cancer-immunogenic information and can prime naive T cells to cytotoxic T cells within a short time. Second, the CAPRI principle seems to be efficient in all types of cancer, since CAPRI cells can destruct carcinomas of the breast, colon, lung, ovary, stomach, adrenal cortex, melanoma and sarcoma cells (Table 1). Third, the CAPRI cell therapy can be started 1 week after the isolation of immune cells from the peripheral blood. Since CAPRI cell therapy is efficient in patients without cytokine infusions, no toxic side effects have been observed.

In vitro generation of HLA-unrestricted immune effector cells against cancer: the concepts of LAK, MAK, MAK-DC & CIK methods

Several strategies have been developed to enhance the observed ability of immune cells *in vitro* to destroy cancer cells. The lymphokine-activated killer (LAK) method uses IL-2 to stimulate IL-2-responsive lymphocytes against cancer cells *in vitro*. LAK cells can lyse cancer cells, which do not express certain HLA molecules, but are blocked by those HLA molecules [8]. LAK cells do not attack normal cells; the administration of LAK

Table 1. Successful cascade-primed immune cell lysis of all so-far tested cancer cell types *in vitro*.

Cancer type	Patients (n)	Histopathology	Stages
Breast	10	Infiltrating ductal, multicentric ductal, invasive lobular, DCIS	T-4N0-2M0-1
Colon	1	Adenocarcinoma	T4N2M1
Melanoma	2	Eye, skin	T1, T3
Lung	1	Squamous cell carcinoma	T4
Ovary	4	Endometrioid carcinoma, cystadenomcarcinoma, adenocarcinoma	T3NN0-1M0-1
Basal cell	1	Nodulocystic basal cell carcinoma	–
Skin/vulva	1	Bowman's disease	CIN III
Stomach	3	Mucosa, adenocarcinoma	T1-3N1-3M0-1
Adrenal cortex†	1	Adenocarcinoma	T2NxM1
Muscle†	1	Spindle cell sarcoma	T2b

†Cascade primed effector cells destroyed all tested autologous cancer cells *in vitro* within 24 h nearly completely, however, in the case of adenocarcinoma of the adrenal cortex, the cell cultures had to be supplemented with 125 U/ml IFN-γ and with 5 ng/ml IL-18 in the case of spindle cell sarcoma (muscle) for efficient cancer cell lysis.

cells together with IL-2 is accompanied with very high toxicity. Because they are not HLA restricted, they can lyse allogeneic tumors [9].

The cytokine-induced killer (CIK) method can be considered as a more developed version of the LAK method, as in addition to IL-2, IL-1, IFN-γ and the anti-CD3 monoclonal antibody are used for stimulation. Expanded CIK cells are NK-like T lymphocytes (NKT) and express CD3 and CD56. They are similar to LAK cells, but not HLA restricted. A further improvement of the cytotoxic capacity of CIK cells has been reported by adding a combination of IL-2 and IL-15 to the cultures in the stimulation period [10]. CIK cells seem to be suited to fight remaining resistant leukemic cells after reinstallation of autologous bone marrow. Since CIK cells are not HLA restricted, they can be infused also into allogeneic patients [11,12].

An important disadvantage of LAK and CIK cells is the need for high doses of IL-2. Although the high toxicity of IL-2 has been recognized early in the clinical setting [8], IL-2 has to accompany the infusions of NK and NKT cells. As described later, depletion of NK and/or NKT $CD56^+$ cells from CAPRI cells does not significantly decrease the efficiency of cancer cell destruction by CAPRI cells [13]. Since addition of IL-2 in *in vitro* tests did not enhance the lysis of cancer cells by CAPRI cells, IL-2 has not been given to patients treated with CAPRI cells.

The macrophage-activated killer (MAK) and MAK-dendritic cell (MAK-DC) methods differ from the LAK and CIK methods as activated macrophages are employed as killer cells rather than $CD3^+$ NKT lymphocytes. Therefore, other cytokines are used for stimulation: IFN-γ for stimulating MAK cells and GM-CSF, IL-13 or vitamin D3 for stimulating MAK-DCs. MAK cells have been inserted locally to sites of metastatic ovarian cancer or into the bladder of 17 patients with bladder cancer [14,15]. MAK cells cannot be used as information cells for the priming of naive T cells against cancer cells, whereas in the CAPRI cell method, monocytes serve as information cells and do not show significant cytotoxicity of cancer cells. MAK and MAK-DCs are poor stimulatory cells in the allogeneic mixed lymphocyte reaction and are not able to stimulate $CD3^+$ cytotoxic T cells. To improve this lack of stimulatory capacity, lipopolysaccharide was added to the cell cultures. This enhanced the stimulatory capacity of MAC-DC, but the expression of CD80, CD83, CD86 and HLA class II remained unmodified in both MAK and MAK-DCs. Therefore, MAK-DCs could induce cytotoxic $CD3^+CD8^+$ T cells against MART-1-expressing melanoma cells only via HLA class I [16,17]. However, it is now recognized that substantial cytotoxic actions against cancer cells require $CD3^+CD4^+$ T cells, which support cancer attacks of cytotoxic $CD3^+CD8^+$ T cells by cytokine production and direct cytotoxicity. The depletion of various immune cell populations from PBMCs before and during the cascade priming procedure revealed the crucial role of $CD3^+CD4^+$ T helper cells. Cancer cell destruction by $CD3^+CD8^+$ cytotoxic T cells was only minimal without the help of $CD3^+CD4^+$ T cells. [13].

Gene variants of the HLA complex are responsible for the presentation of different cancer-immunogenic peptides, which induce the specific immune reactions against different cancer types

The histocompatiblity antigens of the human MHC were discovered with antibodies, which agglutinated leukocytes [18]. If lymphocytes of two individuals were cocultured in the mixed lymphocyte culture, lymphocytes did not react against autologous lymphocytes or against lymphocytes from a sibling, who carried the same HLA antigens [19]. This seemed to confirm that immune cells cannot react against autologous cells or cells expressing the same HLA antigens. However the existence of autoimmune diseases indicates that immune cells can react against autologous cells and these diseases were found to be associated with particular HLA antigens [20]. A clear demonstration of a destructive autoimmune attack is Type I (juvenile) diabetes. Here cytotoxic T lymphocytes destroy the insulin-secreting islet cells of the pancreas, preferably in individuals with certain HLA variants. Why the adaptive immune system started these attacks was unclear. Some details of the enigma were resolved in principle with the discovery of a peptide in the crystallized HLA-A2 molecule, which immediately illuminated the sophistication of the HLA system. This showed that the HLA molecule was only a frame for the presentation of self or microbial peptides. Therefore, the immune system can distinguish cells expressing HLA molecules with microbial (disease) peptides from cells expressing self peptides. Thus, it appeared to be useful to identify immunogenic cancer peptides in order to stimulate cytotoxic T lymphocytes. However, numerous variants (i.e., alleles) exist for most of the classical HLA genes, which present peptides to lymphocytes in the groove of the HLA molecule. For example, 1448 different HLA-A proteins have been detected so far and a similar diversity of allotypes (proteins) has been detected for HLA class I molecules HLA-B (1988 variants) and HLA-C (1119 variants) [21]. Each individual expresses only two of these 1448 HLA-A protein variants and two of HLA-B, HLA-C and the HLA class II genes. A high degree of polymorphism exists also for the HLA class II genes. It is important to realize the impact of this diversity for the cellular immunotherapy of cancer, since each of these HLA proteins will bind and present a different

spectrum of microbial-specific or cancer-type-specific peptides. Several studies showed the deep influence of HLA alleles and allotypes on cancer susceptibility and cancer resistance [22–26]. Even polymorphisms of other nonclassical HLA genes in the HLA chromosomal segment, for example, the immunoproteasome, which processes self and microbial peptides for presentation by the HLA molecule, significantly influence susceptibility to cancer [27]. Which immunogenic cancer peptide should be selected for the stimulation of cytotoxic lymphocytes? The selection of a peptide for a frequent HLA allotype such as the frequently chosen HLA-A2 appears, at first, as a reasonable solution, but bears another problem. Malignant tumors suppress HLA expression or develop HLA class I-negative variants [28]. How can one find, among millions of T lymphocytes, the T-cell clones of the patient, which can recognize cancer-immunogenic peptides presented by the patients' HLA variants? The answer of several investigators was to mix cancer cells and immune cells of the patient outside the body and to support the T lymphocytes with IL-2. In this way, all different T-cell clones are confronted with tumor cells, which hopefully present sufficient immunogenic cancer peptides.

Generation of human cancer-specific T lymphocytes via the antigen T-cell receptor

In one of the first mixed lymphocyte–tumor cell culture experiments, cytotoxic lymphocytes were generated and expanded with IL-2 and the T lymphocytes were subsequently cloned in order to increase their efficiency against the cancer cells. Two T-cell clones showed specific reactions only against autologous melanoma cells [29]. A similar mixed melanoma–lymphocyte culture supplemented with IL-2 was also successful in generating cytotoxic T lymphocytes [30]. However, further expansion of the generated cytotoxic T lymphocytes with IL-2 was, in both reports, not successful. A similar difficulty of expansion was initially observed with TILs. However, if TILs were grown on OKT3- (an antibody against CD3) stimulated allogeneic feeder cells, expansion with IL-2 was successful in some patients [6,7]. A closer look at TILs will point to some reasons for this.

Advantages & disadvantages of TIL therapy

The cytotoxic activity of TILs is clearly directed against their respective autologous cancer cells. TILs do not attack other (normal) cells of the body [6,7]. Several studies have shown that the presence of TILs in the tumor tissue correlates with a good prognosis. Patients with ovarian cancer survived several years longer if the pathologist detected TILs in the removed cancer tissue [31]. But why is the tumor not eradicated by TILs? The more recently favored explanation is tumor editing [32]. TILs are able to eliminate some cancer cells that express certain antigens, but not cancer cells that express other cancer antigens. Thus, the tumor seems to change its antigenic profile. Several studies detected a change of the antigenic profile of malignant cells even in the blood plasma after chemotherapy or radiation [33–36]. However, one would expect that fresh/naive lymphocytes from the blood would again infiltrate a recurring tumor and recognize the changed antigenic peptide. The loss of immunogenic cancer cells and the rise of nonimmunogenic cancer cells could be one reason for the time-limited success of TILs against the malignant tumor. However, additional important features of cancer cells impede their recognition by immune cells. Most cancers originate from epithelial or connective tissues. They are not professional APCs. TILs find a nonstimulating environment with the danger of inactivation for numerous reasons: cancer peptides are not or inappropriately presented by weakly expressed HLA molecules, or costimulatory molecules for the activation of $CD4^+$ T helper cells are absent. There is also a lack of HLA class II expression by most cancer cells. Furthermore, many cancer cells produce interleukins, especially IL-10 and TGFβ, which downmodulate immune responses. The addition of IL-2 to the TIL therapy modality supports cytotoxic T lymphocytes, but cannot turn epithelial cells into professional APCs. As described later, CAPRI cells do stimulate HLA class II expression of cancer cells, which in turn induces $CD4^+$ T helper cells.

There are other practical obstacles. Usually, only few TILs can be isolated from a tumor. Therapy with TILs requires a fairly long time for expansion in order to obtain sufficient cell numbers. Nevertheless, Rosenberg and his group have succeeded in generating high numbers of TILs from melanomas. The number of remissions in melanoma patients was significantly increased, if the TIL therapy was combined with radiation and chemotherapy in order to destroy inactivated TILs and refill the space with freshly *ex vivo*-expanded TILs [7]. TILs represent an important progress, but does have its drawbacks.

The concept of the CAPRI procedure against cancer

All cells which express HLA molecules can present autologous, as well as microbial or cancer-associated peptides via HLA class I molecules [37–43]. However, professional APCs are required in order to differentiate T cells into cytotoxic T-effector cells and to induce the help of $CD4^+$ T cells [44–47]. Blood monocytes can either differentiate in tissues to macrophages or in the blood to DCs. DCs seem to be the primary cells that can prime cytotoxic $CD8^+$ T cells via HLA class I and

CD80/CD86 and CD28 engagement, as well as $CD4^+$ T helper cells via HLA class II and CD40-CD40L engagement. Since proteins of malignant tumors and even newly arising mutated proteins can be identified in the blood [37–43], it was plausible to try to stimulate APCs to achieve an optimal presentation of the cancer proteins that they have ingested. The stimulated APC can activate and differentiate naive T lymphocytes into effector cells, which recognize and destroy cancer cells. The methods of cascade priming are described in detail elsewhere [13]. Here, we discuss the implication of the cascade-priming steps for the maturation of cancer-specific effector cells and the contribution of monocytes, $CD3^+CD8^+$ T lymphocytes, $CD3^+CD4^+$ T lymphocytes and DCs to the priming procedure and lysis of cancer cells.

The first step of cascade priming: stimulation of the APC with activated T cells

Instead of trying an array of different cytokines for the stimulation of APCs, the natural conversation partners for APCs, the T lymphocytes, were used to stimulate the APC. T lymphocytes were not separated from peripheral blood mononuclear cells (PBMCs). To achieve a maximum production of T-cell cytokines, T lymphocytes were activated with the CD3 antibody OKT-3 (Figure 1A). The presence of APCs and the addition of IL-2 prevents activation-induced T-cell death by the OKT3 antibodies, which are immobilized in plastic flasks. The result of this stimulation is depicted in Figure 1B.

The second step of the priming cascade: activation of APC, downmodulation/ internalization of the CD3–T-cell receptor complex

The CD3 molecule is a hexameric complex consisting of δε and γε chains, a pair of ζ chains or a ζυ. CD3 associates with the αβ-T-cell receptor (TCR) and transmits the signals from the αβ-TCR into the interior of the cell. Stimulation of CD3 is sometimes falsely equated with the stimulation of the αβ-TCR by antigen. Ligation of CD3 with the OKT3 antibody activates all $CD3^+$ T cells, but cannot differentiate naive T cells into effector cells. The desired effect of the OKT3 CD3 ligation is the production of cytokines, which enhances the expression of the HLA class I and II peptide complexes, but which also causes a downmodulation or internalization of the CD3–TCR complex (Figure 1B) [48,49]. This means that T cells cannot recognize the peptides presented by HLA class I and class II molecules of the APC and cannot mature into T effector cells. Therefore, naive/resting T lymphocytes (not separated from PBMCs) are added in the next step.

The third step of the CAPRI procedure: initiation of naive/resting T-cell maturation to effector cells by activated monocytes & the initiation of monocyte differentiation

Additional PBMCs from the patient's blood, containing naive T cells expressing the αβ-TCR, are added to the culture. Activated monocytes express HLA class I and class II molecules. Activated monocytes differentiate the naive/resting T cells from the newly added PBMCs into effector cells in the CAPRI procedure (Figure 1C) because the depletion of monocytes completely prevented the maturation of the added naive T lymphocytes to T effector cells [13]. This absolute requirement of monocytes for priming against cancer proteins raises the question of whether cancer proteins block the differentiation of monocytes into DCs. Depletion of DCs in this step reduced the lytic capacity against cancer cells by approximately 50% [13]. DCs cannot initiate the priming of naive/resting T cells in the absence of monocytes, but their presence enhances the destruction of cancer cells in all phases of priming as fewer CAPRI cells are required for cancer cell destruction [13]. Blocking antibodies against HLA class I or class II showed that the cancer-immunogenic information presented to T cells occurs via HLA molecules, that is, it is HLA restricted [13].

The fourth step: differentiation of monocytes to DCs

DCs are considered to be the best at presenting microbial and cancer peptides to T lymphocytes. They constitutively express peptides via their HLA class I and class II molecules and can, in this way, stimulate cytotoxic T cells and helper T cells. DCs are therefore often used in the cancer therapy, but they have to be prepared for this purpose either by the incorporation of cancer peptides or by other genetic manipulations. Surprisingly, monocytes are the prime presenting cells in the CAPRI procedure and are able to mature naive/resting T cells to T effector cells (Figure 1D). The rapidity of differentiation of many monocytes to DCs within 24 h is even more surprising. The dynamics of the process were studied with phenotype markers, activation markers and expression of HLA class I and class II. For this, monocytes were removed from PBMCs, labeled with green fluorescence (carboxyfluorescein succinimidyl ester) and resuspended to the PBMCs before initiating the CAPRI procedure. An increasing number of fluorescence-marked monocytes lost the $CD14^+$ phenotype and expressed the CD83 marker with further increase after 5 days [13]. The activation markers CD80, CD86 and CD40, as well as HLA class I and class II molecules also showed enhanced expression on the fluorescence-marked cells, which increased further

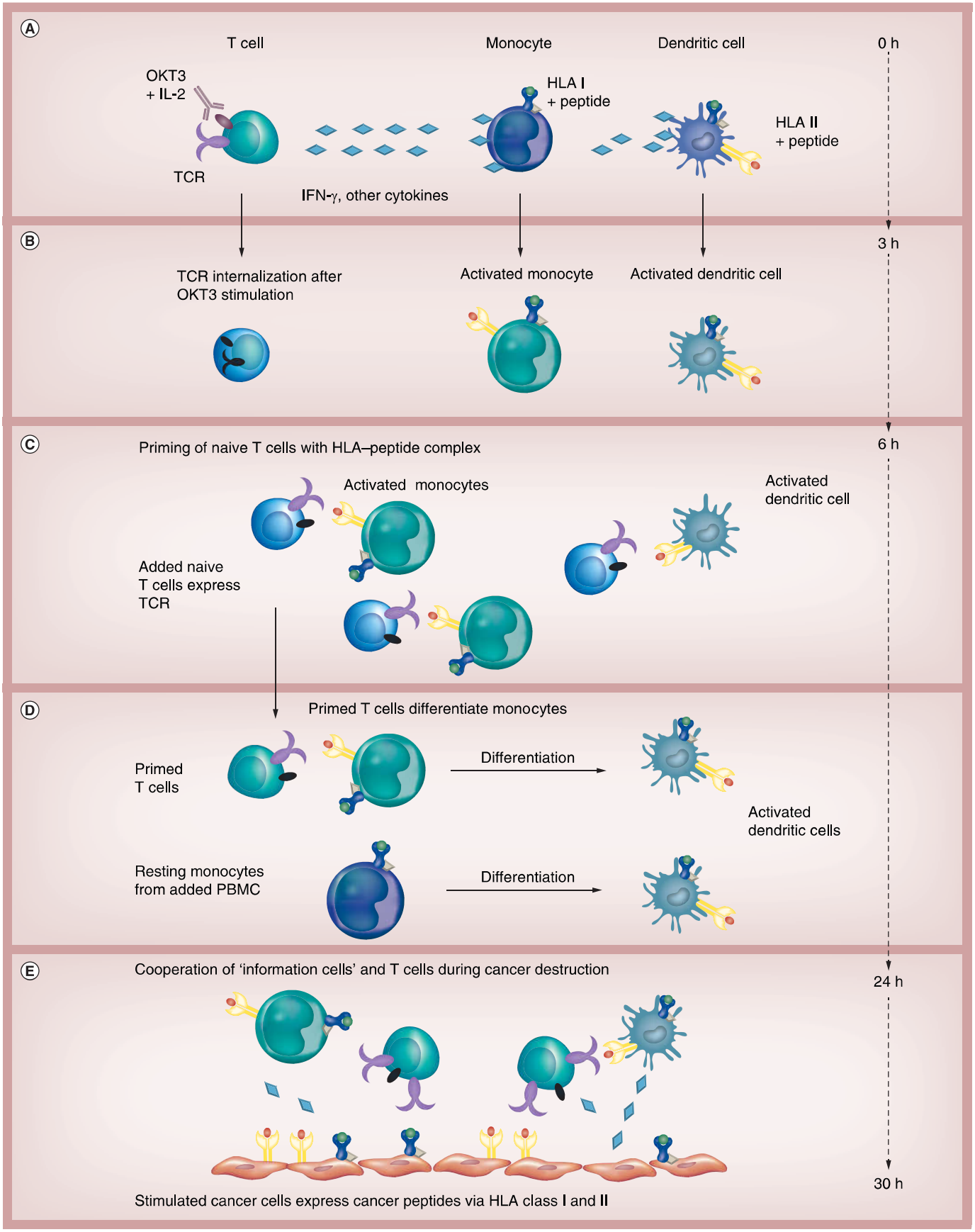
A
T cell
Monocyte
Dendritic cell
0 h
OKT3
+ IL-2
TCR
HLA I
+ peptide
HLA II
+ peptide
IFN-γ, other cytokines
B
3 h
TCR internalization after
OKT3 stimulation
Activated monocyte
Activated dendritic cell
C
6 h
Priming of naive T cells with HLA–peptide complex
Activated monocytes
Activated
dendritic cell
Added naive
T cells express
TCR
D
Primed T cells differentiate monocytes
Primed
T cells
Differentiation
Activated
dendritic cells
Resting monocytes
from added PBMC
Differentiation
E
Cooperation of 'information cells' and T cells during cancer destruction
24 h
30 h
Stimulated cancer cells express cancer peptides via HLA class I and II

Figure 1. Four activation steps of cascade priming and of the cancer destruction phase (see facing page). **(A)** T-cell activation with OKT3 antibodies activates bystander APCs in PBMC cultures (blue). **(B)** CD3 ligation by the OKT3 antibody downmodulates/internalizes the αβ-TCR. **(C)** Activated monocytes and some dendritic cells instruct and stimulate naive/resting T cells via the αβ-TCR. **(D)** Two possible pathways of monocyte differentiation to dendritic cells: αβ-TCR-activated T cells differentiate monocytes to dendritic cells by contact and/or cytokines. **(E)** T-effector cells and dendritic cells induce expression of HLA class I (blue) and HLA class II (green) on cancer cells.
PBMC: Peripheral blood mononuclear cell; TCR: T-cell receptor.

at day 5. Therefore, the costimulatory molecules, which are essential for activation of cytotoxic T cells and helper T cells, as well as enhanced expression of HLA class I and class II molecules, which present the cancer-immunogenic peptides, were expressed by the rapidly maturing monocytes. The enhanced signaling by costimulatory and HLA molecules by stimulated monocytes is, together with cytokines, probably the main factor for the rapid development of the cytotoxic capacity of the T lymphocytes. Another very important factor, the influence of the CAPRI cells on the expression of HLA molecules by the cancer cells, is discussed in the next section.

The fifth step: the destruction of cancer cells by CAPRI cells

The destruction of cancer cells is a joint enterprise of the CAPRI cell quartet, which consists of monocytes, DCs, T helper cells and T cytotoxic cells.

Two interactions of CAPRI cells with cancer cells during the phase of destruction appear to be major factors for the success of CAPRI cells in the destruction of cancer cells (Figure 1E). First, increased expression of HLA class I and class II molecules by cancer cells after contact with CAPRI cells drives cytotoxic T effector cells to destroy cancer cells. Second, DCs and monocytes provide help during cancer cell destruction. Depletion of DCs from CAPRI cells before the initiation of cancer cell lysis reduced the success of destruction by 50% [13]. It is not clear whether the help of DCs is provoked by cancer cell contact and acts by cytokine production or by providing costimulatory signals, which would prevent inactivation of effector T cells. The help of DCs could also come from T-cell signals after cancer cell contact. T cells could demand support of DCs by recruitment of further naive/resting cells.

CAPRI cells in a preclinical mouse model

In a preclinical study, human breast cancer cells were implanted into 12 nude mice. After the infiltrative growth of the cancer cells in all 12 mice, CAPRI cells of the patient were injected into six mice and unstimulated immune cells of the patient into the other six mice. The tumor grew in mice that did not receive CAPRI cells, to an average size of 3 cm in diameter. In the group that received CAPRI cells, the tumors grew to an average size of 0.5 cm in diameter. The average survival time of mice receiving CAPRI cells was 43 days, and 30 days in mice receiving autologous resting immune cells (negative control). In summary, mice treated with CAPRI cells survived significantly longer and showed a significant suppression of tumor growth [13].

Results of clinical cancer case series

Female patients with breast cancer represent the largest number of cases treated with CAPRI cells. Two groups of patients are depicted (Figures 2 & 3): patients with distant metastases and patients without distant metastases. The curves show months of survival after diagnosis. All female breast cancer patients attending the immunotherapy center and treated with at least 500 million CAPRI cells were included in the statistical analysis without further selection. These patients were compared with cancer patients of the same stage from the Munich Tumor Center [13]. Survival of the female breast cancer patients of the Munich Tumor Center does not significantly differ from those published in text books, for example, in '*Conn's Current Therapy 2010*' [50]. It is important to note that the recommended modality of treatment was a continuous treatment twice or three-times weekly for 6 months with 60–80 million CAPRI cells per injection. Depending on the success of treatment, which was evaluated by tumor markers or radiologic examinations, the frequency of cell injections was reduced to one injection every second or third week. However, some patients did follow this regimen and others did not. Patients were treated in an adjuvant fashion, which introduces variables of chemotherapy and radiation modalities. It cannot be determined whether these standard modalities were supportive or detrimental to the CAPRI cell therapy. Nevertheless, adjuvant treatment with CAPRI cells of breast cancer patients with distant metastases resulted in an average survival of 53 months compared with 31 months of the patients with the same tumor staging and standard therapy of the Munich Tumor Center. The difference between standard treatment and standard treatment plus adjuvant therapy was evaluated after 5 years and was highly significant ($p = 4.53 \times 10^{-9}$; Figure 2). A survival advantage was also observed for breast cancer patients with no distant metastases treated with CAPRI cells compared with patients of the Munich Tumor Center with standard therapies ($p = 1.192 \times 10^{-4}$; Figure 3).

Only small groups of patients with other types of cancer were treated with CAPRI. Of special interest

are patients with non-small-cell lung cancer (NSCLC) stages IIIB or IV because only few patients survive the median of 1 year. Of four patients with NSCLC in stages IIIB and IV, two died with stage IV after 39 months and 41 months; the other two with stage IIIB have now survived 55 and 120 months without any sign of recurrence [51]. It should be mentioned here that the median survival of patients with IIIB differs only minimally from patients with stage IV [52].

A case report from China described the direct injection of CAPRI cells into the pleural cavity of a patient with lung metastasis of a collecting duct carcinoma resulting in several immediate improvements (CAPRI cells were here called modified CIK cells) [53].

First results from an ongoing case-controlled study at the University of Kunming are shown in Table 2 and Figure 4 and present results of 30 patients with lung cancer at different stages treated with CAPRI cells and untreated matched patients (Table 2). After a 1-year observation period, a significant advantage can be seen in patients treated with CAPRI cells (Figure 4). There is a follow-up of the described patients and further recruitment of new patients [Song X, Unpublished Data]. This confirms the favorable results seen in four German patients with NSCLC with stages IIIB and IV [51].

The claim that CAPRI can probably be used for all types of cancer rests on the observation that CAPRI cells destroyed cancer cells from the respective patients in *in vitro* experiments with the same efficiency as has been observed with breast cancer patients and lung cancer patients (Table 1) [13]. Whereas one method of CAPRI cell preparation could be used for most types of cancer, the adenocarcinoma of the adrenal cortex was more susceptible to CAPRI cells cultured with IFN-γ and the spindle cell sarcoma was more susceptible to CAPRI cells cultured with the addition of IL-18 (Table 1).

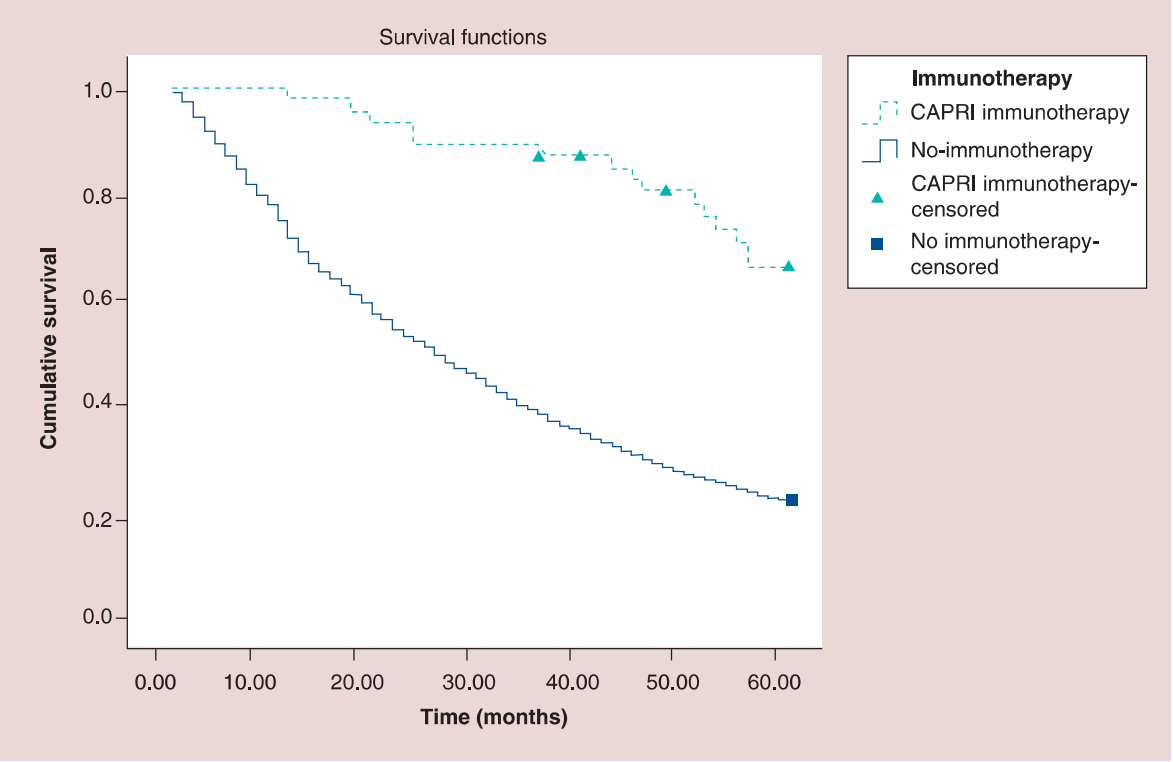

Figure 2. Survival curve of breast cancer patients with distant metastases with adjuvant cascade-primed immune cell therapy and no cascade-primed immune cell therapy. All patients with distant metastasis were included independent from tumor size, lymph node metastasis or tumor grading if they received at least 500 × 10^6 CAPRI cells in 5 years. Most patients, however, received more CAPRI cells, and most patients followed our suggested schedules. We recommended a schedule of 60–80 × 10^6 CAPRI cells three-times per week for 6 months, followed by injections twice weekly for another 6 months. In most patients, the therapy was continued with an injection every second or third week for the next 4 years. The 5-year survival curve for patients treated with CAPRI cells (n = 46) was compared with patients from the Munich Tumor Center (n = 1801) without CAPRI therapy in the same tumor stages. Each patient (T1-4N0-2, G2-3) with diagnosed distant metastasis (M1) was included in the analysis, if receiving at least 500 × 10^6 CAPRI cells. Kaplan–Meier analysis is shown, log-rank (Mantel-Cox; $\chi^2 = 34.383$; $p = 4.35 \times 10^{-9}$). The average survival time of patients with CAPRI therapy was 53.16 ± 1.91 months compared with 30.54 ± 0.49 months for patients without CAPRI immunotherapy.
CAPRI: Cascade-primed immune.
Reproduced with permission from [13].

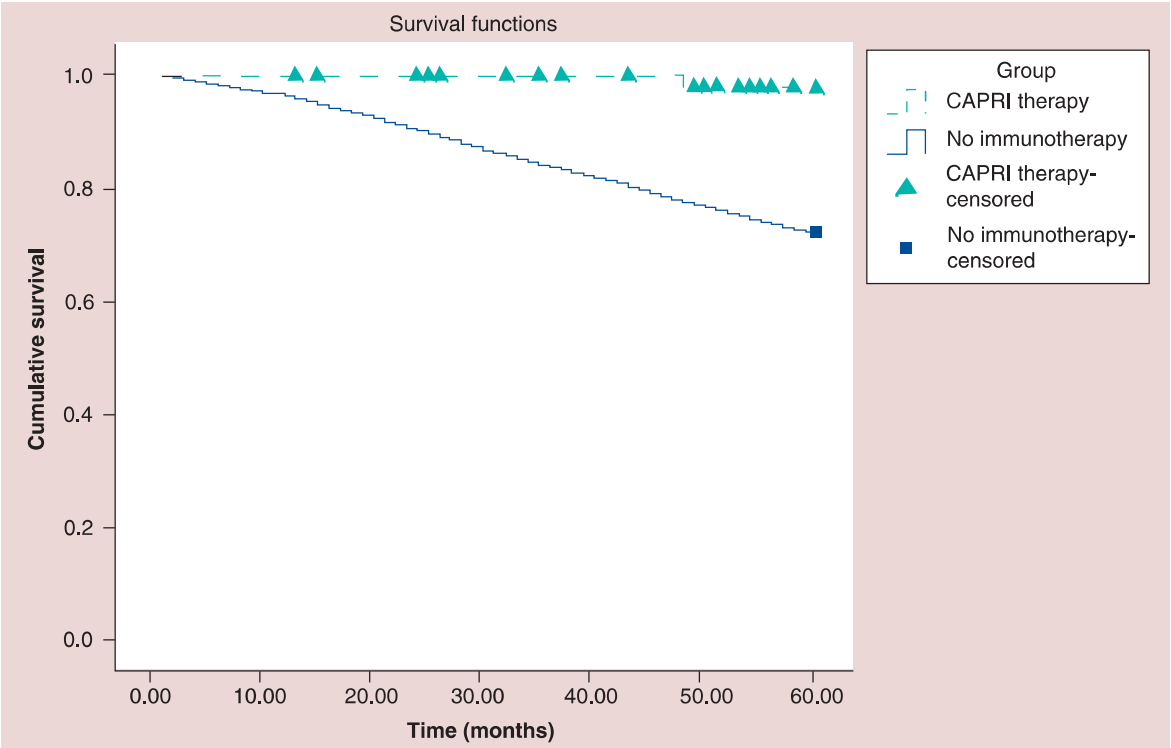

Figure 3. Survival curve of breast cancer patients without distant metastases with adjuvant cascade-primed immune cell therapy and no cascade-primed immune cell therapy. All patients without distant metastasis were included, independent from tumor size, lymph node metastasis or tumor grading, if they received at least 500 × 10^6 CAPRI cells in 5 years. Most patients, however, received more CAPRI cells, and most patients followed our suggested schedules. We recommended a schedule of 60–80 × 10^6 CAPRI cells once a week for 1 year. In most patients, the therapy was continued with an injection every second or third week for the next 4 years. The 5-year survival curve of patients treated with CAPRI cells (n = 59) was compared with patients from the Munich Tumor Center (n = 9821) without CAPRI therapy in the same tumor stages. Each patient (T1-4N0-2, G2-3) without metastasis (M0) was included in the analysis if receiving at least 500 × 10^6 CAPRI cells. Kaplan–Meier analysis is shown, log rank (Mantel-Cox; χ^2 = 14.805; p = 1.192 × 10^{-4}). The average survival time of patients with CAPRI therapy was 59.75 ± 0.25 months compared with 52.31 ± 0.15 months for patients without CAPRI immunotherapy. CAPRI: Cascade-primed immune.

The clinical impact of CAPRI cells & other ACT methods in the therapy of cancer

The straightforward technique of the CAPRI procedure without genetic manipulation and without the need for defining cancer-immunogenic peptides, as well as the efficiency of cancer destruction by CAPRI cells makes them a favorite among other ACT methods. Another important aspect of each successful ACT method is the balance of chemotherapy and/or radiation therapy with the maintenance of functioning immune cells. The finding of immune cells in removed ovarian cancer tissue and its correlation with a prolonged survival of several years [31] is an obligation to test, in future, the function of lymphocytes from the peripheral blood after chemotherapy or radiation, as well as the numbers of leukocytes. CAPRI cell therapy will profit from destruction of big tumor masses by chemotherapy and radiation. In the future, it should be possible to balance the attacks of standard therapeutical modalities with the maintenance of functioning immune cells.

Conclusion

Novel ACT with CAPRI cells significantly increased the survival rate of patients with breast cancer, with and without metastases, and also of patients with NSCLC [13,51]. The life quality in these patients was maintained. The efficiency of CAPRI cells is based on several factors: first, on monocytes that were, for the first time, identified as cells harboring immunogenic cancer information and activated with activated T cells; second, on lymphocytes of the peripheral blood that were not damaged by encounter with cancer cells, which can inactivate T cells by rudimentary signaling [54]; third, on the cooperation of cytotoxic $CD8^+$ and $CD4^+$ T cells, which were stimulated together, allowing supporting interactions ('help') during priming and cooperative cytotoxicity against cancer cells; fourth, on continuous therapy, as CAPRI cells are given repeatedly over many years; and finally, on the advantage that 'tumor-edited' cancer cells and their products will also be ingested and processed by monocytes. This means that lymphocytes can be primed years

Table 2. First results of an ongoing case-controlled study of lung cancer patients: basic clinical information from the tumor hospital of the Yunnan province, University Kunming.

Patients	Control group	CAPRI cell-treated group
Sex (female/male)	20/10	19/11
Average age ± SD years	58.9 ± 8.9	59.4 ± 10.2
Stage		
T1N0M0	5	4
T1N0M1	0	0
T1N1M0	0	0
T1N1M1	1	1
T2N0M0	1	2
T2N0M1	0	0
T2N1M0	11	10
T2N1M1	1	2
T3N0M0	2	2
T3N0M1	1	0
T3N1M0	3	2
T3N1M1	5	7
Squamous/adeno	18/12	19/11
OP status		
After OP	22	19
No OP	8	11
Chemotherapy		
Completed	28	27
No chemotherapy	2	3
Radiation therapy		
Completed	21	22
No radiation therapy	9	8

No statistical differences were observed between CAPRI group and control group.
Adeno: Glandular-type epithelial formation; CAPRI: Cascade-primed immune; OP: Surgery; Squamous: Scaled-type epithelial formation.

later against the new cancer variants, underlining the value of a continuous CAPRI cell therapy.

Although the efficiency of CAPRI cells has been so far only shown in case series of breast cancer patients and in NSCLC patients, the *in vitro* tests of CAPRI cells against autologous cancer cells of other tissues listed in Table 1 suggest that CAPRI cells will be effective in most types of cancer. However, studies with more patients in the clinical setting need to prove the broad applicability of the CAPRI method.

Future perspective

The CAPRI procedure is currently only used in the Immunotherapy Research Center in Munich and in seven university hospitals in China. It should be possible to simplify the preparation of CAPRI cells and spread the technique to many hospitals in Europe.

Development of closed culture systems for the production of CAPRI cells

In China, CAPRI cells are produced in good manufacturing practice rooms and require all staff for control of the facility and for production. It should be possible to develop culture systems that do not require specific good manufacturing practice rooms, for example, using closed culture systems. In this way, CAPRI cells could be produced in many hospitals at low costs. This cost aspect is of paramount importance for the social systems, if one considers that in Germany alone 200,000 new cancer patients are diagnosed annually.

Elution of immunogenic cancer peptides from activated monocytes

Monocytes activated by CD3-activated T cells digest cancer proteins and differentiate naive/resting T cells

to successful cytotoxic effector cells. Since the presentation is HLA restricted, the immunogenic cancer peptides are presented by HLA molecules. It should be possible to elute such peptides from the HLA molecules as previously suggested from HLA molecules of HIV-resistant individuals [55]. Alternatively, phagosomes may also serve as a source of immunogenic peptides [56]. These cancer-immunogenic peptides could be sequenced and may give clues on specific microbial factors in different cancers.

Production of monoclonal antibodies against cancer

The activated monocytes or the above-described isolated cancer-immunogenic peptides could be used for immunization of rats and mice to produce truly cancer-immunogenic monoclonal antibodies. Although monoclonal antibodies were not very successful so far in solid cancers, a set of antibodies against several cancer-specific epitopes could be more successful in cancer destruction. Such highly cancer-specific antibodies could be also useful to detect metastatic cancer cells.

Analyses of cytokines produced 'in time'

The tight time window of the different steps during cascade priming suggests that different cytokines are produced to stimulate or to downregulate the stages of activation, which results in the birth of aggressive CAPRI cells. With the help of microarray analyses, it could be possible to identify cytokines or combinations of cytokines which could be used in the therapy of cancer and also chronic diseases.

Financial & competing interests disclosure

R Wank holds International, European and US patents for the CAPRI procedure. The authors have no other relevant affilfiations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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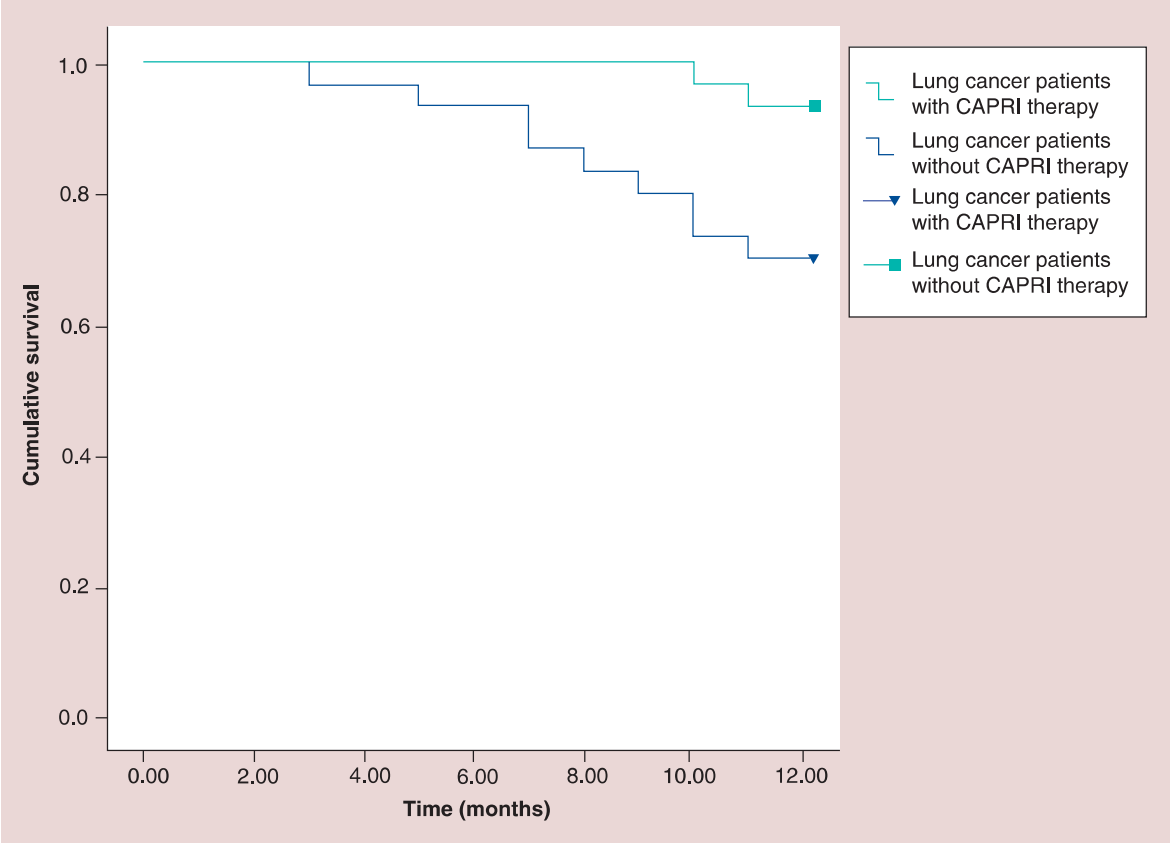

Figure 4. Kaplan–Meier test of survival rate in lung cancer patients treated with cascade-primed immune cells compared with lung cancer patients not treated with immunotherapy. In the patient group treated with CAPRI cells, the 12-month average survival time was 11.90 ± 0.072 months. In the patient control group, the 12-month average survival time was 10.73 ± 0.043 months. Log rank (Mantel-Cox) test showed $\chi^2 = 5.76$, $p = 0.016$. After 1 year follow-up, CAPRI therapy indicated an increase survival time for the lung cancer patients. The study will be continued with more patients and the patients will be followed-up during the next years.
CAPRI: Cascade-primed immune.

Executive summary

Background

- Cascade-primed immune (CAPRI) cell therapy is rapidly applicable and efficient in most cancer types. It uses cancer information presented by monocytes to differentiate T cells to cytotoxic cells.

Immune cell therapy of cancer was considered futile for a century

- Do cancer cells differ enough from healthy cells of the body?
- Microbial influence in cancer development was not appreciated.

***In vitro* generation of HLA-unrestricted immune effector cells against cancer**

- Lymphokine-activated killer cells require additional (toxic) IL-2 infusions for therapy.
- Macrophage-activated killer cells can directly attack cancer cells. They do not present cancer-immunogenic peptides.

Gene variants of the HLA complex are responsible for the presentation of different cancer-immunogenic peptides, which induce the specific immune reactions against different cancer types

- Many diseases are associated with HLA variants.
- Numerous HLA variants exist in the population.
- Different HLA molecules present different peptides: own, microbial-derived and cancer-derived.

Generation of human cancer-specific T lymphocytes via the antigen T-cell receptor

- Successful generation of tumor-specific T-cell clones was possible, but not further expansion.

Advantages & disadvantages of TIL therapy

- Only few TILs invade the tumor and tumor products can damage TILs. Therefore, it is difficult and time consuming to obtain sufficient numbers of TILs for therapy.
- Tumor tissues express HLA only weakly; cancer-immunogenic peptides are poorly presented.

The concept of CAPRI procedure against cancer

- Stimulation of professional APCs is a solution of the predicament to select one cancer-immunogenic peptide from numerous different peptides. Autologous monocytes present the 'best' cancer-immunogenic peptides for the respective individual.

The first step of cascade priming: stimulation of APC with activated T cells

- Anti-CD3 activated T lymphocytes were used to stimulate APCs.

The second step of the priming cascade: activation of APC, downmodulation/internalization of the CD3–T-cell receptor complex

- OKT3-stimulated T cells activate expression of cancer-immunogenic peptides of APCs, but internalize the T-cell receptor antigen.

The third step of the CAPRI procedure: initiation of maturation of naive/resting T cells to effector cells by activated monocytes & initiation of monocyte differentiation

- Activated monocytes with enhanced HLA expression (class I and II) differentiate naive/resting T lymphocytes via the T-cell receptor to effector cells.

The fourth step: differentiation of monocytes to dendritic cells

- Monocytes rapidly differentiate into dendritic cells during the CAPRI procedure. During the differentiation the enhanced signaling by co-stimulatory and HLA molecules, as well as cytokines result in rapid development of cytotoxic effector cells.

The fifth step, the destruction of cancer cells by CAPRI cells

- Contact between CAPRI and cancer cells induces an increased expression of HLA molecules on cancer cells, which boosts the cytotoxic capacity of the effector T cells. Dendritic cells and monocytes seem to augment cytotoxic activity.

CAPRI cells in a preclinical mouse model

- Implantation of human breast cancer cells and injection of autologous CAPRI cells into nude mice significantly reduced the size of the growing tumor and prolonged the animals' survival time compared with a control group.

Results of clinical cancer case series

- CAPRI therapy in female breast cancer patients of all stages resulted in significantly enhanced survival rates compared with a control panel of the Munich Tumor Center. Four patients with non-small-cell lung cancer survived many years longer than the expected median survival of 1 year.

The clinical impact of CAPRI cells & other ACT methods in the therapy of cancer

- The straightforward technique of the CAPRI procedure makes it to a favourite among ACT methods. No genetic manipulation and no search of cancer-immunogenic peptides are required.

Conclusion

- Case series in breast cancer, non-small-cell lung cancer and *in vitro* experiments with several cancer types showed that CAPRI cell therapy currently seems to be the most effective and easy to accomplish ACT. It does require neither defined immunogenic peptides nor genetic manipulations.

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