 Activated Monocytes Prime Naïve T Cells Against Autologous Cancer: Vigorous Cancer Destruction In Vitro and In Vivo

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Abstract
It has not been considered so far that antigen-presenting cells (APC) may phagocytose immunogenic material from autologous cancer cells. Assuming the presence of cancer-immunogenic material in APC, we developed a novel autologous priming method that does not require tumour cells or identified peptides. Cancer-immunogenic information came from CD14+ monocytes. When stimulated with CD3-activated T cells, monocytes primed CD3+-CD4+ and CD3+CD8+ resting/naïve T cells to become powerful effector cells within 24 h. During priming, depletion of CD14+ monocytes but not CD1a+CD83+ dendritic cells prevented T cell priming. During cancer cell destruction, dendritic cells, but not monocytes, enhanced cancer cell lysis. The cascade-primed (CAPRI) immune cell quartet comprising monocytes, dendritic cells, CD4+ T and CD8+ T cells induced a significant decrease in the number of suppressive CD25highFoxP3+CD4+ T cells. CAPRI cells induced a marked upregulation of MHC class I and class II expression in cancer cells, which is crucial for autoimmune-like lysis. We show in vivo evidence of the CAPRI cell concept in nude mice. In humans, we present circumstantial clinical evidence showing the efficacy of CAPRI cells in an adjuvant treatment attempt for breast cancer patients with metastasis (N = 42). Compared to patients at the Munich Tumor Center (no CAPRI treatment N = 428), almost double the expected number of patients survived 5 years (P = 1.36 × 10−14). The CAPRI method is a safe procedure without negative side effects. High numbers of cancer-specific CAPRI cells can be obtained within a week against different cancer types for efficient adoptive cell therapy.

Introduction
Numerous therapeutic modalities have been developed to hinder the growth or induce the destruction of malignant tumour cells. The multitude of modalities reflects the inexhaustible number of strategies that cancer cells use to evade control by immune cells. However, as of yet unrecognized immune responses must prevent the rise of carcinoma cells in women carrying resistance-associated immune response genes of the HLA system [1–5]. Immune surveillance of cancer growth by T lymphocytes necessarily includes the recognition of tumour-immunogenic peptides. To present such peptides to T cells, dendritic cells have been incubated with tumour cell lysates, pulsed with defined tumour peptides or transfected with RNA or DNA from tumour cells [6, 7].

Gene mutations and their corresponding mutated cellular proteins can serve as tumour markers. For example, mutations of the p53 gene have been identified in free circulating DNA in precancer and cancer patients [8, 9]. Cytotoxic T cell responses to different and differently mutated tumour targets have been reported [10–16]. We have been interested in identifying conditions that would stimulate antigen-presenting cells (APC) to process, express and transfer tumour-immunogenic information to naïve T cells, leading to their maturation to T effector cells, to prevent their inactivation, as has been observed in tumour-infiltrating lymphocytes [17, 18].

Antigen-presenting cells were stimulated by activating T cells in PBMC cultures with the monoclonal antibody OKT3. Because ligation of CD3 chains by OKT3 antibodies downmodulates the CD3/ζ/αβTCR complex via
internalization or by preventing their recycling [19, 20], we added unstimulated autologous PBMC as a source of naïve T cells expressing the αβ TCR.

Here, we show that MHC-restricted efficient cancer cell lysis by cascade-primed (CAPRI) cells results from the cooperation of a cellular quartet consisting of T helper cells, T cytotoxic cells, dendritic cells and monocytes that upregulate and induce MHC class I and class II expression in cancer cells.

Finally, we provide preclinical and circumstantial clinical evidence for the CAPRI concept by showing efficient and significant lysis of cancer cells in nude mice and in patients with different cancers in an adjuvant treatment attempt.

Materials and methods

Tumour samples and establishment of autologous tumour cell lines. Immune cells and autologous tumour samples were donated by informed and consenting patients referred by doctors for the support of radiation or chemotherapy with adjuvant adoptive immunotherapy (ACT). The tumour samples were used to establish cancer cell lines to provide a control for analysing the lytic capacity of activated immune cells. The ethics recommendations of Helsinki with subsequent amendments of Tokyo 1975, Hong Kong 1989 and Somerset West 1996 were followed.

Tumour samples were minced to small pieces and cultured in 50-ml flasks using supplemented tumour culture medium (RPMI 1640 with l-glutamine, supplemented with 10% FCS, TNEAA, G5 Supplement; all from PAA, Coelbe, Germany) and optimized culture conditions. Unused tumour samples were also minced to small pieces and cryopreserved in DMSO, like PBMC [21]. The establishment of cell lines that divided at least 20 times was successful only with samples from patients who had not yet received chemotherapy or radiation therapy. All cell lines originated from Caucasian patients.

Isolation of immune cells. PBMC were isolated from venous blood puncture or leukapheresis samples by density gradient centrifugation as described previously [21] using lymphocyte separation medium (LSM; PAA). Immune cells were either used immediately or cryopreserved and stored in the nitrogen gas phase. Isolation, cryopreservation and thawing procedures as well as the use of optimized culture conditions (38.5 °C, 6.5% CO2) have been described in detail [21].

Activation of T cells in PBMC bulk cultures: CD3 activation and CAPRI cell generation. Both methods started with the activation of T cells in PBMC bulk cultures using the CD3 monoclonal antibody OKT3 (Orthoclone; Cilag, Sulzbach-Taunus, Germany), which binds to the non-polymorphic ε-chain of the CD3 molecule, and the addition of interleukin 2 (IL-2; Proleukin; Chiron, Ratigen, Germany). CD3 antibodies were immobilized at a concentration of 1 μg/ml in 0.05 M borate buffer pH 8.6 and distributed in 50-ml tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). Coated flasks were kept at 4 °C at least overnight and washed twice with phosphate-buffered saline prior to incubation with PBMC. PBMC were added at a concentration of 2 × 10⁶ cells/ml in a total volume of 10 ml, and IL-2 was added within 2–12 h at a concentration of 20 U/ml. CD3-activated cells were expanded on day 4 with IL-2 (20 U/ml) and harvested on day 7 for immediate use or cryopreservation.

For the generation of CAPRI cells, CD3-activated PBMC were removed from the flask after 4–6 h, washed and then cocultured in a second CD3 ‘antibody-free’ flask with an equal number of unstimulated autologous PBMC, which contained naïve/resting T cells, at a concentration of 2 × 10⁷/ml in a total volume of 10 ml. Cells were expanded on day 1 with IL-2 (20 U/ml) and harvested on day 4.

Microscopic classification, preparation of tumour target cells and quantification of cancer cell destruction using the Cr⁵¹-release assay. Cancer cells were removed from flasks by trypsinization, resuspended in culture medium (RPMI 1640 with l-glutamine; PAA) supplemented with 10% FCS and washed twice. Cancer cells were counted and distributed in different concentrations into 96-well flat-bottom culture plates (Falcon; Becton Dickinson, Heidelberg, Germany) either for microscopic evaluation of lysis or for the Cr⁵¹-release assay. Before the addition of effector cells, cancer cells were allowed to recover overnight to restore their membrane integrity after trypsinization. Reorganization of cancer cells into a coherent cell layer better reflected the situation in vivo and significantly lowered the rate of spontaneous Cr⁵¹ release, an as yet unresolved problem of the Cr⁵¹-release assay for measuring tumour cell destruction.

The Cr⁵¹-release assay was performed in duplicate at varying effector to target ratios using 2 × 10⁷ target cancer cells. Maximum Cr⁵¹ release was determined using labelled target cells, and spontaneous release was discerned by incubating target cells in medium alone. The per cent of spontaneous release was calculated as follows: (spontaneous cpm:maximum cpm) × 100; the per cent of cytotoxicity was calculated as follows: [(experimental cpm – spontaneous cpm):(maximum cpm – spontaneous cpm)] × 100. Quantitative lysis of cancer cells using the Cr⁵¹-release test was assessed after 5–6 h and after 18–22 h by following the ‘classical’ guidelines for the cell-mediated lympholysis (CML) assay [22] with the crucial difference in tumour target preparation described earlier. Representation of data followed classical CML papers [23–27].

The degree of lysis was estimated by microscopic inspection after 18–24 h. The scale used corresponded to conventional HLA microscopic estimated evaluation. This
scale designates a lysis of more than 80% as strong positive (+ +), 60–79% as positive, 40–59% as weak positive, 20–39% (+) as doubtful positive and <20% (−) as negative (Fig. 2).


Imaging medium: RPMI 1640 with l-glutamine (PAA) supplemented with 10% FCS; pictures were taken at room temperature.

Figures were prepared with Microsoft® Powerpoint® 2000 (9.0.2716) and corel PHOTO-PAINT, version 12.0.0.536.

To determine the influence of HLA class I and II molecules on cancer lysis, monoclonal antibodies were added at the start of the CAPRI cell/cancer cell cocultures. The antibody W6/32 (1 µg/ml) (Abcam, Cambridge, UK) was used to block HLA class I, and L243 (1 µg/ml) (Abcam) was used to block HLA class II.

Depletion of CD3, CD4, CD8 and CD14 positive subpopulations from PBMC with magnetic beads. Mouse anti-human CD3, CD4, CD8 and CD14 conjugated to magnetic beads; CD14 negative isolation kits and Pan Mouse IgG beads were obtained from Dynal (Invitrogen, Paisley, UK) and used according to manufacturer’s instructions.

The manufacturer’s depletion protocol was repeated three times for a depletion efficiency >98%.

CD4+ T cells were isolated from CD3-isolated populations to spare CD14+CD4+ monocytes.

Tracing of monocytes during CD3 and CAPRI stimulation. About 40 × 10^6 PBMC were obtained from each donor, 20 × 10^6 PBMC were treated with Dynabeads® Untouched™ human monocytes kit (Invitrogen) to obtain CD14+ monocytes, according to manufacturer’s instructions.

Purified CD14+ monocytes were labelled with CFSE (eBioscience, San Diego, CA, USA) according to manufacturer’s instructions. About 20 × 10^6 PBMC were depleted of CD14+ monocytes by Dynabeads® CD14 (Invitrogen) according to manufacturer’s instructions. CFSE-labelled CD14+ monocytes were added to the CD14-depleted PBMC to reconstitute a PBMC population with CFSE-labelled CD14+ monocytes. The reconstituted PBMC were stained with anti-CD14 PE, HLA-DR-PE, CD1a-PECy5.5, with anti-CD40-PECy5.5, CD80-PECy5.5, CD83-PECy5, CD86-PECy5.5 and with anti-HLA-A,B,C-PECy5.5 to trace the phenotype of CFSE-labelled CD14+ cells during CD3 stimulation or during the CAPRI procedure.

Flow cytometry. Expression of cell surface markers was determined by flow cytometry using the Becton-Dickinson FACScan analyzer and CELLQUEST software (Becton-Dickinson). CD14+ cells were CFSE-labelled to trace the changes in phenotype. In brief, cells were harvested and stained with anti-CD14 PE, HLA-DR-PE, CD1a-PECy5.5, with anti-CD40-PECy5.5, CD80-PECy5.5, CD83-PECy5, CD86-PECy5.5 and with anti-HLA-A,B,C-PECy5.5 to trace the phenotype of CFSE-labelled CD14+ cells during CD3 or CAPRI stimulation. For the analyses of cell surface markers on CD3-stimulated and CAPRI cells, cells were collected and stained with anti-CD3-FITC, CD14-PE, CD19-PECy5.5, with anti-CD3-FITC, CD4-PE, CD8-PECy5.5, with anti-CD3-FITC, CD14-PE, CD56-PECy5.5 and with anti-CD3-FITC, CD16-PE, CD56-PECy5.5. For Foxp3 staining, cells were stained first with anti-CD4-PE, fixed, permeabilized with human Foxp3 staining buffer set and then stained with FITC-anti-human Foxp3.

The conjugated mouse monoclonal antibodies were obtained from BD Biosciences or eBioscience. The human Foxp3 staining buffer set was obtained from eBioscience.

Presence of CD4+ T lymphocytes could not be replaced in the priming phase or in the cytotoxicity assay by supernatants from CAPRI cell cultures. CAPRI culture supernatants were added to CAPRI cell cultures to clarify whether CD4+ T lymphocytes provided only ‘cytokine help’ to cytotoxic CD8+ T cells or participated as effector cells in cancer cell destruction. To avoid the depletion of CD14+CD4+ monocytes, CD3+ cells were first isolated from PBMC cultures (1), and then CD4+ cells were depleted. The CD4+-depleted CD3 isolate was added to (1).

Supernatants were added before CD3 activation or to unstimulated PBMC, which were added in the second step to supply T cells expressing the z/βTCR.

Cytotoxicity testing of human CAPRI cells against autologous breast cancer cells in nude mice. Animal experiments were authorized by the ethic committee of the University of Wuhan, China, and designed by S. Gu and performed at the Wuhan University under the supervision of S. Gu.

Twelve 6-week-old nude female mice (BALB/c-nu) were obtained from Wuhan University, Center for Animal Experiments, China. Cells from the breast cancer cell line ZB (raised 10 years ago from one of the patients in the Institute of Immunology of the University of Munich and in culture since then) were washed in PBS and resuspended in PBS at a concentration of 1 × 10^7/ml. Mice were injected subcutaneously with 1 × 10^6 breast cancer cells in 0.1 ml of PBS. Mice of the control group (n = 6) were injected with 1 × 10^6 autologous PBMC, and ve- rum group mice (n = 6) were injected with 1 × 10^6 autologous CAPRI cells every second day until day 15. PBMC and CAPRI cells were introduced surrounding the injected tumour locations. Mice were observed for 45 days after cancer cell injection. Tumour size was measured for the first time after 21 days. Mice were killed if the maximum tumour diameter was >15 mm unless the tumour killed the mouse before that point. After 45 days, the experiment was completed, and all mice were killed.
Results

Cancer cell lysis by CAPRI cells within 24 h

We stimulated APC in PBMC bulk cultures by activating T cells with the OKT3 antibody, which binds to a conformational epitope expressed by CD3-ε or CD3-εβγ chains [29], and added unstimulated PBMC to CD3-activated PBMC as a source of T cells expressing the zβTCR. Microscopic inspection indicated little or no reduction in cancer cell numbers after 24 h of coculture with CD3-activated PBMC (Fig. 1A) compared with carcinoma cultures at time zero (Fig. 1A, B), but most cancer cells were lysed after being cocultured with CAPRI cells (Fig. 1F). In chromium release assays, CD3-activated PBMC showed no significant lytic activity (Fig. 1G), while CAPRI cells lysed 27.1% of cancer cells at a 5:1 effector to target (E:T) ratio and 89.9% of cancer cells at a E:T ratio of 20:1 (Fig. 1G).

CAPRI cells lysed MHC-restricted cancer cells

The generation of cytotoxic T cells depends on interactions between the αβ TCR and the pMHC [30]. MHC restriction was analysed using allogeneic cancer cells and antibodies blocking the pMHC. CAPRI cells from two unrelated breast cancer patients with defined HLA class II DQ alleles were tested along with breast cancer cells from six unrelated patients (Fig. 2A). After 24 h, CAPRI cells lysed the autologous cancer cells robustly and lysed the cancer cells with shared HLA-DQ1 alleles approximately half as well, whereas a lack of HLA-DQ sharing resulted in minimal background lysis (Fig. 2A). This suggests that HLA class II surface molecules on APC presented tumour-immunogenic peptides, but complete lysis may depend on the sharing of both HLA class I and class II antigens. This was indirectly supported by the observation that cancer cell lysis was blocked with HLA class I and class II antibodies. Lysis was strongly reduced with the antibody W6/32 binding to all HLA class I molecules and the antibody L243 binding to HLA class II molecules (Fig. 2B, C). Both antibodies, W6/32 and L243, block the lysis of cancer cells significantly; (B) W6/32: \( P_{\text{slope}} = 2.49 \times 10^{-9}, P_{\text{intercept}} = 6.52 \times 10^{-9} \), L243: \( P_{\text{slope}} = 2.50 \times 10^{-9}, P_{\text{intercept}} = 4.70 \times 10^{-9} \). (C) W6/32: \( P_{\text{slope}} = 6.04 \times 10^{-9}, P_{\text{intercept}} = 4.58 \times 10^{-9} \), L243: \( P_{\text{slope}} = 9.19 \times 10^{-10}, P_{\text{intercept}} = 2.16 \times 10^{-9} \).

Isotypic control antibodies do not block the lysis of cancer cells by CAPRI cells. Figure 2B, patient 1: \( P_{\text{slope}} = 0.504, P_{\text{intercept}} = 0.572 \), Fig. 2C, patient 2: \( P_{\text{slope}} = 0.881, P_{\text{intercept}} = 0.678 \).

The required concurrence of HLA class I and class II presentation indicates a comprehensive interdependence of helper and cytotoxic T cells for the successful lysis of cancer cells. CAPRI cells showed very weak activity against the NK target cell K562, which usually does not express HLA antigens (data not shown), perhaps because K562 lysis is usually mediated by activated NKT cells in PBMC cultures [31].

CAPRI cells enhanced HLA class I and class II surface expression in epithelial and other solid cancer cells

How can CAPRI cells lyse cancer cells in an HLA-restricted manner, despite cancer cells tending to downregulate HLA expression [32, 33]? We compared the HLA expression of CFSE (5(6)-carboxy fluorescein diacetate N-succinimidyl ester)-labelled cancer cells after coculture
with autologous unstimulated PBMC, only CD3-activated PBMC or CAPRI cells using a low effector to target ratio of 5:1 (Fig. 3). CAPRI cell-stimulated cancer cells showed a 40% increase in mean fluorescence intensity (MFI) in HLA class I expression (MFI versus MFI) and a 60% increase in HLA-DR class II expression (MFI versus MFI) (Fig. 3A). The enhanced MHC class II expression in cancer cells could be pivotal for the destructive power of CAPRI cells, as CD4 interactions augment cytotoxic T cell responses [34, 35].

Maturation of monocytes to dendritic cells during the CAPRI procedure

Stimulated APC express high levels of MHC class I and class II molecules along with B7 and other costimulatory molecules [36]. We analysed phenotypic markers of CFSE-labelled CD14+ monocytes before activation (day 0) and 1 day (day 1) and 5 days (day 5) after activation (Fig. 4). In CAPRI cells, a considerable number of monocytes lost CD14 expression and matured, as defined by the acquisition of the dendritic cell markers CD1a and CD83 at day 1 and their marked upregulation at day 5 (Fig. 4B). Upregulation of the costimulatory molecules CD80, CD86 and CD40, and HLA-DR class II and HLA class I molecules was also observed (Fig. 4B). In only CD3-activated PBMC, the number of CD14+ monocytes and cells expressing CD83 and CD1 remained constant. Upregulation of the costimulatory molecules CD80, CD86, CD40 and HLA class I and of HLA-DR was clearly lower than in CAPRI cell cultures (Fig. 4C). Quantitative analysis of leucocyte subpopulations in CD3-activated PBMC and CAPRI cells from five patients with cancer showed significantly more matured dendritic cells in CAPRI cultures than in CD3-activated PBMC (paired t-test, \( P = 0.000096 \)) (Table 1) and a higher percentage of monocytes in CD3-activated PBMC compared to CAPRI cells on day 5 (paired t-test, \( P = 0.023 \)) (Table 1).

Depletion of CD14+ monocytes, CD83+ dendritic cells, CD3+CD8+ or CD3+CD4+ T cells and cancer cell lysis

Depletion of subpopulations and the resulting effect on lysis were analysed at the following time points: 1) in
unstimulated PBMC before CD3 activation; 2) in unstimulated PBMC to be added to CD3-activated PBMC, and 3) from CAPRI cells before coculture with cancer cells (Fig. 5). Depletion of CD3⁺CD8⁺ T lymphocytes at each time point prevented CAPRI cells from developing any lytic capacity (Fig. 5D), and depletion of CD3⁺CD4⁺ T cells had the same effect at each time point (Fig. 5C). Depletion of CD14⁺ monocytes at time point 1) or 2) completely abrogated the lytic activity of CAPRI cells (Fig. 5A), whereas depletion of monocytes at time point 3) did not significantly influence the lysis of cancer cells.

Depletion of CD83⁺ dendritic cells reduced the development of CAPRI cell lytic efficiency by 50% (Fig. 5B). This ‘medium’ contribution to the lytic capacity of CAPRI cells may indicate a continuous supply of contact information and/or of cytokines to T effector cells during cancer cell destruction. The failure of immune responses as a consequence of rudimentary immunogenic information from cancer cells has been previously demonstrated [32, 33]. Depletion of B lymphocytes did not significantly influence lysis (data not shown).

Presence of CD4⁺ T lymphocytes versus replacement by CAPRI culture supernatants

CAPRI culture supernatants should clarify whether CD4⁺ T lymphocytes only provide cytokine help to cytotoxic CD8⁺ T cells. Supernatants were added at depletion time point 1) or 2). In the absence of CD4⁺ T cells, cancer cells were only minimally destroyed (not shown).

Less increase in the regulatory T cell population (CD4⁺CD25⁺Foxp3⁺) in CAPRI cell cultures

Several reports have described the suppression of cytolytic responses against human cancer cells by CD4⁺CD25⁺ regulatory T cells [37–45]. Modulation and suppression have appeared to be restricted to CD4⁺CD25⁺Foxp3⁺ T lymphocytes, either antigen-specific or non-antigen-specific [37–45]. The percentage of CD4⁺CD25⁺Foxp3⁺ T lymphocytes is strongly increased in CD3-activated cells compared to unstimulated PBMC. In CAPRI cultures, this increase is only moderate (Fig. 6).

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**Figure 2** Cascade-primed (CAPRI) cells lyse MHC-restricted cancer cells: (A) microscopical evaluation of lysis after 24 h at a E:T ratio of 20:1 by using autologous cancer cells and cancer cells sharing or not sharing HLA-DQB1 alleles. Autologous cancer cells were lysed most robustly (++) cancer cells sharing HLA-DQB1*0603 or DQB1*0201 with CAPRI-CTL1 (upper line, HLA-DQB1*0201 and DQB1*0202 express identical surface molecules) or sharing HLA-DQB1*0201 with CAPRI-CTL2 were noticeably lysed (+). No significant lysis occurred without HLA-DQ class II matching; the quantity of lysed cancer cells was estimated to be similar to those in the HLA microcytotoxicity test [28]. (B, C) Evaluation by Chromium 51-release assay and by blocking with HLA class I (W6/32, block the lysis of cancer cells significantly; (B) W6/32: \( P_{\text{intercept}} = 2.49 \times 10^{-9} \), \( P_{\text{slope}} = 6.52 \times 10^{-9} \), L243: \( P_{\text{intercept}} = 2.50 \times 10^{-9} \), \( P_{\text{slope}} = 4.70 \times 10^{-9} \). (C) W6/32: \( P_{\text{intercept}} = 6.04 \times 10^{-9} \), \( P_{\text{slope}} = 4.58 \times 10^{-9} \), L243: \( P_{\text{intercept}} = 9.19 \times 10^{-10} \), \( P_{\text{slope}} = 2.16 \times 10^{-9} \).
Significant lysis of cancer cells by CAPRI cells in nude mice

Breast cancer cells were implanted in twelve female mice. After tumour implantation, six mice were injected with autologous PBMC (controls), and the other six were injected with autologous CAPRI cells (verum). In this breast cancer model, the average tumour size was 29.64 ± 6.95 mm in the control group, whereas the tumour size was 5.08 ± 1.66 mm in the mice receiving CAPRI cell therapy. Furthermore, the verum group showed an average survival time of 43 ± 1.17 days, and the control group survived an average of 29.67 ± 1.92 days ($P = 5.06 \times 10^{-4}$, Fig. 7A, C, D, Table 2).

CAPRI cell treatment for patients with breast cancer and survival time

Breast cancer patients (T1-4N0-2M1, G2-3) treated with CAPRI cells in an adjuvant treatment attempt were compared with patients of the Munich Tumor Center (T1-4N0-2M1, G2-3) using Kaplan–Meyer statistics. All breast cancer patients with distant metastasis who received at least $500 \times 10^6$ CAPRI cells in total were included in the comparative analysis. It was recommended that patients should receive $60–80 \times 10^6$ CAPRI cells thrice a week for at least 1 year. Despite variations in the frequency of injection and cell number, which are unavoidable in treatment attempts, CAPRI cell-treated patients showed a significant increase in survival (Fig. 7B). Patients reported no adverse reactions from CAPRI cells; rather, adverse reactions from chemotherapy were neutralized by the CAPRI cell therapy. Most patients with adjuvant CAPRI cell treatment were able to resume professional activities 1 day after chemotherapy.

Discussion

The dramatic power of autologous MHC-restricted immune responses, first recognized by Zinkernagel and Doherty [46], contrasts with the immune surveillance failure of MHC-restricted tumour-infiltrating lymphocytes (TIL). However, TIL can be successfully revived in vitro [47]. ACT using autologous TIL combined with non-myeloablative chemotherapy and irradiation achieved a complete response in seven of 25 patients (28%) [47], a fundamental progress for ACT.
Unprofessional presentation of tumour-immunogenic peptides and costimulatory molecules by cancer cells often induces the inactivation of naïve T cells. To avoid this inactivation, we developed the following priming procedure: we stimulated APC by activating T cells in PBMC bulk cultures with the OKT3 anti-human CD3 antibody to enhance the expression of MHC and costimulatory molecules (Fig. 4). To ensure the transfer of MHC information, resting naïve T cells expressing high levels of the zβ TCR were added because CD3 activation downmodulates the zβ TCR [19, 20]. The highly efficient lysis of autologous cancer cells by these CAPRI immune cells (Fig. 1G) confirmed our notion that stimulated APC of patients with cancer harbour/present sufficient tumour-immunogenic information to generate T effector cells. The nearly complete blocking of lysis with antibodies against HLA class I and class II molecules demonstrated the MHC restriction of the lysis (Fig. 2B, C). Furthermore, lysis of allogeneic cancer cells was more efficient when CAPRI cells and cancer cells shared HLA class II antigens (Fig. 2A).

To assess the expression levels of costimulatory and MHC molecules of activated APC, we labelled CD14+ monocytes with CFSE (Fig. 4). In CAPRI cultures, but not in CD3-activated PBMC, labelled monocytes showed an increased expression of CD40, CD80, CD86 and HLA-DR and HLA-DR and HLA class II antigens (Fig. 4). Particularly interesting was the numerical decrease in CD14+ monocytes and the numerical increase in CFSE-labelled cells with the CD1a+CD83+ mature dendritic cell phenotype, which was not seen in CD3-activated PBMC (P = 0.000096, Fig. 4A–C, Table 1).
To determine the contribution of CAPRI cell subpopulations during priming and lysis, we depleted subpopulations from PBMC before CD3 activation, from unstimulated PBMC before their addition to previously activated PBMC or from CAPRI cells before cancer cell lysis (Fig. 5). Depleting either CD8+ T cells or CD4+ T cells at any time point prevented cancer lysis (Fig. 5). Supernatants from undepleted CAPRI cell cultures did not rescue the effect of CD4+ T cell depletion, indicating a significant cytotoxic activity of CD4+ T cells (not shown). The ‘unrealized potential’ of CD4+ T cells for cancer ACT has been proposed and evaluated [48, 49].

Depletion of APC populations revealed that CD14+ monocytes but not dendritic cells were absolutely required for priming. Monocytes could not be removed from PBMC cultures before CD3 activation or from unstimulated PBMC before their coculture with CD3-activated PBMC. One might speculate that capture of tumour material may silence monocytes in vivo and prevent their differentiation to dendritic cells. Until now, failing immune responses have been explained mainly by the inactivation of T cells at the tumour site rather than by mute monocytes. We do not know whether activated monocytes, activated monocytes in transition of differentiation or rather de novo matured dendritic cells are the crucial cells required to prime naive T cells. Differentiation of monocytes here may have been induced by activated monocytes priming naive T cells, and primed T cells could drive monocyte differentiation to dendritic cells. The discovery of monocytes as the primary cells responsible for passing tumour-immunogenic information to T cells could perhaps be used to differentiate monocytes with cytokines in vivo to prime naive T cells into tumour-specific effector T cells.

Depletion of dendritic cells from CD3-activated PBMC or from unstimulated PBMC reduced cancer cell destruction by approximately 50%. It has been reported that signals from activated CD4+ T cells enable dendritic cells to instruct bystander dendritic cells to prime naive CD4+ T cells [50, 51]. However, CD3-activated T cells could not initiate this dendritic circuit without monocytes; furthermore, monocytes were required in unstimulated PBMC cultures that were added to CD3-activated PBMC.

Depletion of monocytes from CAPRI cells immediately before their coculture with cancer cells did not significantly reduce lysis. However, depletion of dendritic cells decreased cancer cell destruction by 50% (Fig. 5A, B). This suggests that dendritic cells may provide a continuous flow of cytokines and/or of tumour-immunogenic information by building an information bridge between cancer cells and effector T cells to maintain cancer cell destruction by T effector cells. Supplementary professional antigen presentation by activated dendritic cells may prevent rudimentary TCR signalling by cancer cells leading to multiple immunosuppressive effects, such as default secretion of IL-10 by Th1 cells [52].

Taken together, optimal priming for cancer destruction required cell-mediated bidirectional cooperation among a cellular quartet consisting of CD14+ monocytes, CD14+CD1a+CD83+ dendritic cells, CD4+ T cells and CD8+ T cells, whereas a cellular trio comprising dendritic cells, helper T cells and cytotoxic T cells achieved optimal cancer cell lysis without monocytes.

Carcinomas often escape from recognition by down-regulating their own HLA expression [32, 33]. Increased HLA expression of cancer cells correlates with increased survival of patients [53–56]. Could CAPRI cells, which lyse HLA-restricted tumour cells, influence the HLA expression of cancer cells? Examination of CFSE-stained carcinoma cells showed that cocultured CAPRI cells did indeed increase the expression of HLA class I and class II molecules in autologous cancer cells (Fig. 3), and they most likely do so in many cancer types lysed by CAPRI cells (listed in Table 3, lysis not shown). Of particular note was the successful CAPRI cell-mediated lysis of carcinoma cells of Bowen’s disease. These intraepidermally growing carcinoma in situ cells are commonly recalcitrant to therapy because they are enveloped by fibroblasts. Less than 1% of Bowen’s cancer cells bind keratinocyte antibodies in cytopsins (not shown). This cancer is an excellent example of the proposed inhibitory role of tumour stroma, as this stroma can prevent direct lysis by T cells [57]. We interpreted the lysis of Bowen’s cancer cells by CAPRI cells as evidence for cross-presentation and cross-priming between members of the CAPRI cell quartet.
and we also might construe this as evidence for the ability of CAPRI cells to enhance fibroblast processing and presentation of tumour products, which is possible in principle [58].

The observed lower percentage of CD4\(^+\)CD25\(^{\text{high}}\) FoxP3\(^+\) regulatory T cells in CAPRI cultures compared to CD3-activated PBMC (Fig. 6) could augment the cytolytic activity of CAPRI cells. Whereas CD3 stimulation of T lymphocytes favours pathways leading to IL-10-producing cells expressing CD25\(^{\text{high}}\)FoxP3\(^+\)CD4\(^+\) [43], the activation pathway via the αβ TCR [44] may favour the amplification of CD4\(^+\) T cells not expressing FoxP3. Furthermore, activation of dendritic cells during the CAPRI procedure may enhance their ability to abrogate the regulatory activities of CD25\(^{\text{high}}\)FoxP3\(^+\)CD4\(^+\) cells [45].

Our results demonstrate the importance of monocytes and CD4\(^+\) T cells for immune responses against cancer. In the CAPRI procedure, tumour-immunogenic peptides need not be identified and can be presented by (at least)
In three patients, the percentage of CD4\(^+\)/CD25\(^+\)/FoxP3\(^+\) regulatory T cells was compared between PBMC, CD3-activated cells and cascade-primed (CAPRI) cells. In all three patients, the percentage of regulatory T cells in CAPRI cells is slightly higher than in unstimulated PBMC, but much lower than in CD3-activated cells. CD3-activated cells (B) and CAPRI cells (C) of patient 1 were analysed for FoxP3 expression in CD4\(^+\) and CD4\(^+\) cells. A lower percentage of regulatory T helper cells (CD4\(^+\)/FoxP3\(^+\)) was found in CAPRI cells (1.47\%) than in CD3-activated cells (3.58\%). FoxP3 expression was also lower in CD4\(^+\) cells (mostly CD8\(^+\)), 3.22\% in CAPRI cells compared to 11.05\% in CD3-activated cells. The CD4\(^+\)/CD8\(^+\)/FoxP3\(^+\) T cells were supposed to overlap with CD8\(^+\)/CD25\(^+\)/CTLA4\(^+\)/FoxP3\(^+\) regulatory CD8\(^+\) T cells.
Each breast cancer patient (T1-4N0-2, G2-3) with diagnosed metastasis (M1) showed a small but significant increase in the survival times with those of breast cancer patients (T1-4N0-2M1, G2-3) of the Munich Tumor Center (N = 428). The average survival time of the 42 breast cancer patients with breast cancer treated by CAPRI therapy was 55.19 ± 1.68 months. The average survival time of the 428 breast cancer patients with metastasis treated without immunotherapy was 28.60 ± 0.95 months. Kaplan–Meier analysis is shown. Log rank (matel-Cox) \( \chi^2 = 12.09, P = 5.06 \times 10^{-4} \).

The first controlled study with CD3-activated PBMC showed a small but significant increase in the survival rate of patients with hepatocellular carcinoma [60]. The results were interpreted as evidence for the amplification of cancer-specific T memory cells and not effector maturation [61]. This interpretation is compatible with our in vitro results showing marginal lysis of cancer cells by CD3-activated PBMC.

Preclinical evidence of the CAPRI cell concept was obtained by establishing breast cancer tumours in twelve female nude mice. In this breast cancer model, the size of the tumour increased in the control group but was significantly decreased by CAPRI cells \( P = 7.56 \times 10^{-6}, \) Table 2). A significant increase in survival time was also observed for CAPRI cell-treated mice \( P = 5.06 \times 10^{-4}, \) Fig. 6A).

In human patients, circumstantial clinical evidence of the CAPRI cell concept was provided in an adjuvant treatment attempt for breast cancer patients with metastasis (T1-4N0-2M1, G2-3, N = 42) by comparing their survival times with those of breast cancer patients (T1-4N0-2M1, G2-3, N = 428) from the Munich Tumor Center (Fig. 6B). The survival curves of female patients with breast cancer and metastases collected in the Munich Tumor Center are nearly identical with those published in text books like Harrison’s ‘Principles of Internal Medicine’ (7th edition) [62] or Conn’s ‘Current Therapy’ (2010) [63]. Both patient groups received standard combinations of chemotherapy and radiation. The average survival time of patients with adjuvant CAPRI
cell treatment was 55.19 ± 1.68 months; patients receiving only standard therapy survived an average of 28.60 ± 0.95 months (Fig. 6B, P = 1.36 × 10^-14). The standard therapies could have hindered or supported the CAPRI cell therapy. Preventing the growth of huge tumour masses by irradiation or chemotherapy would support CAPRI cell therapy. However, to prevent damage to bone marrow or PBMC, they should be isolated before irradiation or chemotherapy.

In summary, we have shown that a treasure of cancer-immunogenic information is stored only in monocytes and is expressed upon stimulation by CD3-activated T cells. Activated monocytes can prime naïve/resting T cells to become powerful cancer-specific CTL against autologous cancers. We raised CAPRI cells against many different types of cancer (Table 3) and did not find a non-immunogenic cancer. Treatment attempts with CAPRI cells as adjuvant treatment for patients with breast cancer showed that almost double the number of patients survived 5 years, but this needs to be confirmed in standardized clinical studies.

With CAPRI cells, many different cancers can be treated within a week and without negative side effects. Future studies should consider analysing the cytokines secreted by the CAPRI cell quartet at different time periods. Treatment with such cytokines may facilitate the treatment for all patients with cancer in a cost-effective and time-sensitive manner.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sex, age (years)</th>
<th>Primary tumour site</th>
<th>Histopathology</th>
<th>G</th>
<th>TNM stage</th>
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<td>F, 55</td>
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<td>2</td>
<td>T2N1biiiMx</td>
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<td>F, 64</td>
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<td>Infiltrating ductal carcinoma comedocarcinoma</td>
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<td>T1cNxMx</td>
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<td>F, 61</td>
<td>Breast</td>
<td>Multicentric infiltrating ductal carcinoma</td>
<td>2</td>
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<td>Invasive lobular carcinoma</td>
<td>2</td>
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</tr>
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<td>Infiltrating ductal carcinoma</td>
<td>2</td>
<td>T2 pN1bii Mx</td>
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<td>F, 58</td>
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<tr>
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<td>2</td>
<td>T4 pN2 Mx</td>
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<td>T2pNxMx</td>
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<td>T1pNx, R0</td>
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<tr>
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<td>F, 55</td>
<td>Antrum</td>
<td>Adenocarcinoma</td>
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<td></td>
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<td>M, 63</td>
<td>Stomach</td>
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</tr>
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<td>Adrenal cortex</td>
<td>Adenocarcinoma</td>
<td>T2NxM1</td>
<td></td>
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<tr>
<td>SARCi1</td>
<td>F, 42</td>
<td>Muscle</td>
<td>Synovial spindle cell sarcoma</td>
<td>T2b</td>
<td></td>
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</tbody>
</table>

*The described cascade-primed (CAPRI) method needed to be supplemented with IFNγ (125 U/ml culture medium) during the priming phase for successful lysis of the adenocarcinoma of the adrenal cortex.

*With IL-18 for successful lysis of the sarcoma cells (5 ng/ml culture medium).
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Authors’ contributions

Barbara Laumbacher and Rudolf Wank pioneered the CAPRI cell procedure over several years. Songhai Gu designed and performed the elegant FACS experiments. All authors participated in writing the manuscript.

Competing interest statement

Barbara Laumbacher and Songhai Gu have no conflicting interests. Rudolf Wank holds European and International patents for the CAPRI procedure.

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