IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively muster an antigen-independent macrophage response on tumor cells that have shut down tumor antigen expression

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ABSTRACT

During malignant progression cancer cells tend to lose cell surface expression of MHC and other immune antigens, making them invisible to cytotoxic T cells and therefore inaccessible to tumor antigen-directed immunotherapy. Moreover, cancer cell variants that have lost antigen expression frequently contribute to deadly tumor relapses that occur following treatments that had been initially effective. In an effort to destroy antigen-loss cancer cells in tumors, we created a strategy that combines a chimeric antigen receptor (CAR)-redirected T cell attack with an engineered local release of the cytokine IL-12 which recruits and reinforces macrophage function. Cytotoxic T cells were engineered to release inducible IL-12 upon CAR engagement in the tumor lesion, resulting in destruction of antigen-loss cancer cells that would normally escape. Importantly, elimination of the antigen-loss cancer cells was accompanied by an accumulation of activated macrophages that was critical to the anti-tumor response, since removing the macrophages abolished the response and restoring them re-engaged it. Neutralizing TNF-α also abrogated the elimination of antigen-loss cancer cells, implying this pro-inflammatory factor in the process. Taken together, our results show how IL-12 supplementation by CAR T cells can target otherwise inaccessible tumor lesions, in a manner associated with reduced systemic toxicity, by recruiting and activating innate immune cells for a pro-inflammatory response.
Edited Précis:

This study describes a creative and effective cell-based therapeutic strategy to attack antigen-loss cancer cell variants that arise as a result of tumor immunoediting during cancer progression and relapse, employing an antigen-directed T cell immunotherapy that can also muster an antigen-independent macrophage response.
INTRODUCTION

Adoptive cell therapy with antigen-specific cytotoxic T cells has shown efficacy in fighting malignant diseases in pre-clinical models and in first clinical trials (1). T cells of desired specificity or tumor infiltrating T cells are isolated, expanded in culture and re-infused in sufficient numbers to the patient. To make cell therapy more tumor-specific effector T cells are ex vivo engineered to express a T cell receptor (TCR) or chimeric antigen receptor (CAR) with specificity for a tumor-associated antigen. The CAR consists of one polypeptide chain combining the antigen binding site of an antibody with the TCR/CD3ζ signal mediated activating machinery of the T cell (2). So far adoptive cell therapy with genetically engineered T cells has emerged as the most effective treatment for patients with melanoma (3); a number of trials currently explore the treatment of other malignancies.

Despite some remarkable long-lasting tumor regression obtained upon adoptive therapy with antigen-specific T cells in melanoma (4), most immunotherapy trials yield primarily transient tumor regression, likely owing to our neglect of the heterogeneity of tumor lesions at the immunologic level. During progression of the disease, human cancer cells show considerable variability to avoid immune recognition or to disable effector cells, both factors facilitating tumor relapse (5, 6). Antigen processing and presentation processes are frequently defect making cancer cells invisible to T cell recognition. Other mechanisms include an altered cytokine profile and the release of immune repressive cytokines like TGF-β and IL10, the presence of suppressive cells and the in-permissiveness to lymphocyte infiltration. Antigen-loss cancer cells, however, are probably one of the major forces to drive tumor progression despite ongoing specific therapy and to contribute to the majority of cancer deaths. Antigen-loss cancer cells are not affected by antibody-targeted cellular therapies highlighting a
conceptual deficit that cannot be overcome by improving binding affinity, cytolytic activity, amplification, or survival of redirected T cells.

To overcome the deficit we engineered CAR-redirected T cells to secrete inducible IL-12 (iIL-12) upon CAR engaging tumor cells. In those T cells, IL-12 expression is under control of the NFAT₆ minimal promoter that initiates IL-12 transcription upon CAR redirected T cell activation. The main physiological producers of IL-12 are phagocytes and dendritic cells in response to pathogens, to T and NK cell signals, and to components of the inflammatory extracellular matrix (7). IL-12 has a pivotal role in pro-inflammatory and immunoregulatory functions. While IL-12 has no direct effect on tumor cells, the anti-tumor effect is due to improved activation of cytotoxic T cells and NK cells that are the main effector cells of the adaptive and innate immune response in mediating tumor lysis. IL-12 moreover improves the Th1-type helper T cell response, induces a panel of cytokines including IFN-γ and TNF-α, and exhibits anti-angiogenic activities. These privileges explain the considerable efforts to establish IL-12 in tumor therapy. Clinical trials showed some anti-tumor effect of IL-12 with Th1 type responses and infiltration of both NK cells and macrophages in the treated tumor lesion (8, 9). IL-12 therapy, however, is restricted by severe toxicities preventing systemic administration to achieve therapeutic levels in solid tumor lesions (10); for direct IL-12 application, however, most metastatic cancer lesions are not accessible. To accumulate high levels of IL-12 locally in solid tumor lesions, we here made use of the migratory and tissue penetrating capacities of T cells engineered to target the tumor lesion through their CAR and release inducible IL-12 upon CAR engagement. We provide evidence that T cell produced local IL-12 supplement in the tumor lesion attracts activated macrophages that eliminate antigen-loss tumor cells by a TNF-α mediated process.
MATERIAL and METHODS

Cell lines and reagents.

293T (ATCC CRL-11268), LS174T (ATCC CCL 188), Colo320 (ATCC CCL 220.1), C15A3 cells (Dr. M. Neumaier, Universität Heidelberg-Mannheim, Germany). All cell lines were cultured in RPMI 1640 medium, 10% (v/v) FCS (all Life Technologies, Paisly, U.K.). The anti-human IFN-g mAb NIB42 and the biotinylated anti-human IFN-g mAb 4S.B3, the anti-IL12 p40/p70 mAb C15.6 and its derivatives, and the PE-conjugated anti-mouse IL12Rβ1 mAb 114 were purchased from BD Bioscience, San Diego, CA. The Alexa Flour 647-conjugated anti-mouse CD11a mAb M17/4, the Pacific blue-conjugated anti-mouse CD11b mAb M1/70, the Alexa Fluor 488-conjugated anti mouse CD18 mAb M18/2, the PerCP/Cy5.5- and Alexa Fluor 488-conjugated anti mouse CD80 mAb 16-10A1 and the PE/Cy7- and Alexa Fluor 647-conjugated anti-mouse CD86 mAb GL-1 were purchased from BioLegend, San Diego, CA. The PerCP-Cy5.5-labeled anti mouse CD11b mAb M1/70 was purchased from BD. The FITC-conjugated anti-mouse CD86 mAb GL1 was purchased from eBioscience.

Cell sorting.

T cells were isolated from peripheral blood by magnetic activated cell sorting (MACS) using human CD3+ Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Murine macrophages from peritoneal cavity were stained by an Alexa Fluor 488 labeled anti-F4/80 mAb A3-1 (Biozol, Eching, Germany) and isolated by FACS sorting using a BD FACS Aria III.

CAR modification and activation of T cells.
The generation of the retroviral expression cassettes for the CEA-specific CAR BW431/26scFv-Fc-CD3ζeta and the CD30-specific CAR HRS3scFv-Fc-CD3ζ was described in detail (30). To generate the retroviral expression cassette for the pSIN-NFAT₆-IL-12 vector, the NFAT recognition sequence was amplified from pSIN-(NFAT)₆-eGFP vector (31) by PCR, using the following set of primer oligonucleotides (BglII and BamHI restriction sites are underlined): 5’GGGCAGATCTAAGCTTGATATCGAATTAGG3´ (sense) and 5’CCGAGGATCCAGGAGTTGAGGTTACTGTGA3´ (antisense). The constitutive CMV promoter/enhancer LTR sequence was replaced by the amplified NFAT₆ DNA sequence. In a two-step transduction procedure, T cells were retrovirally transduced (32) with the iIL-12 expression cassettes and positively selected in presence of 0.5 mg/ml Geneticin (G418: Sigma-Aldrich, Taufkirchen, Germany) and IL-2 (500 IU/ml) on plates pre-coated with anti-CD3 mAb OKT-3 and anti-CD28 mAb 15E8. Geneticin-resistant clones were transduced with the CEA-specific CAR. CAR expression was monitored by flow cytometry utilizing an PE-conjugated anti-hIgG1 antibody and an FITC-conjugated anti-CD3 mAb (UCHT-1) using a FACS Canto cytofluorometer (BD). IFN-γ in the culture supernatant was recorded by ELISA using matched pair antibodies (clones NIB 42 and B133.5). Mouse IL-12 was bound by anti-mouse IL-12 mAb (clone 9A5) and detected by biotinylated anti-mouse IL-12 (clone C17.8) (BD).

To monitor the cytolytic activity, increasing numbers of macrophages were cocultivated with tumor cells for 24 h in 96-well plates. The specific cytotoxicity of macrophages was monitored by (XTT) based colorimetric assay (Roche Diagnostics, Mannheim, Germany).

Mouse studies and in vivo bioluminescence imaging.

The Governmental Animal Care and Use Committee approved all mouse studies (approval no 8.87-50.10.35.08.071). We used the NIH-III mouse (LystᵇFloxn₁muBtk>Edit) (Charles River, Wilmington, MA) which is deficient in NK, B and T cells. CEA⁺ C15A3 tumor cells (9x10⁵
cells/mouse) were subcutaneously co-injected together with engineered T cells (2x10^5 T cells/mouse) into NIH-III mice (6-7 mice per group). Alternatively, tumors were induced by subcutaneous injection of C15A3 tumor cells and T cells were applied by intravenous injection at day 6. Tumor growth was daily monitored by external measurement using a digital caliper. For bioluminescence imaging, C15A3 and MC38 cells were genetically modified with the click beetle (CB) and the renilla (R) luciferase, respectively. For in vivo imaging D-luciferin (1,5 mg/mouse) or benzyl-coelenterazine (100 µg/mouse) (PJK GmbH, Kleinblittersdorf, Germany) as substrate for the CB and R luciferase, respectively, was intraperitoneally injected. Bioluminescence was recorded using a Photon Imager bioluminescence device (Biospace Lab, Paris, France) equipped with the Photo Vision software (BioSpace) for data post-processing. The exposure time was 300 sec for all recordings. The threshold of bioluminescence signals was automatically determined using the Photo Vision software. Bioluminescence signals were accordingly filtered. Regions of interest (ROIs) were defined as regions above threshold and automatically gated by appropriate program tools. There was no manual gating of ROIs in order to avoid any incoherence. Photon emission intensity (photon/s/sr) was calculated from data of emitted photons from the respective ROI using the Photo Vision software.

**TNF-α neutralization.**

Tumors were induced by subcutaneous co-injection of C15A3 (CEA+) and click beetle luciferase-marked MC38 (CEA-) tumor cells (each 5 x 10^5 cells) at day -6. Tumor bearing NIH-III mice were intraperitoneally injected with the neutralizing rabbit anti-mouse TNF-α antibody MPG-XT3 (150 µg/mouse) (Upstate Biotechnology, Lake Placid, USA) at days -1, 0, +3, +5, +7. The isotype-matched rabbit IgG antibody RTK2071 of irrelevant specificity (Biolegend, San Diego, USA) served as control. T cells expressing anti-CEA CAR and iIL-12 were intravenously injected at day 0 (5 x 10^5 cells per mouse).
Myeloablation and substitution with macrophages.

C57BL/6-Rag2<sup>tm1Cgn</sup>/J mice lacking functional T and B cells were treated with myeloablative concentrations of fludarabine phosphate (150 mg/kg body weight) (Gry-Pharma, Kirchzarten, Germany) and cyclophosphamide monohydrate (200 mg/kg body weight) (Sigma-Aldrich, St. Louis, MO, USA) to deplete NK cells and macrophages. At day 3 after treatment no NK cells or macrophages were detected in the peripheral blood. 293T cells engineered to constitutively express IL-12 (10<sup>6</sup> cells) were subcutaneously injected into pre-treated mice together with CEA<sup>-</sup> MC38 tumor cells (1.5 x 10<sup>6</sup> cells) with or without freshly isolated F4/80<sup>+</sup> macrophages from C57BL/6 mice (2 x 10<sup>5</sup> cells). Control groups were co-injections into pre-treated mice without macrophages, application of 293T cells without IL-12 expression and mice without pre-treatment.

Immuno-histological analyses.

For the detection of cytotoxic macrophages, cryostat sections were stained with the Alexa Flour 647-conjugated anti-CD11a mAb M17/4, with the Pacific blue-labeled anti-CD11b mAb M1/70 and the Alexa Fluor 488-conjugated anti-CD18 mAb M18/2. IL-12 receptor β-chain expression was detected by the PE-conjugated mAb 114. CD80 was detected by the Alexa Fluor 488-conjugated mAb 16-10A1, CD86 by the Alexa Flour 647-labeled mAb GL-1. The staining specificity was assayed using isotype-matched control antibodies. Slides were analyzed using the Carl Zeiss Axiovert 400 M microscope (Karl Zeiss, Oberkochen, Germany).
RESULTS

Peripheral blood T cells were engineered with pre-defined specificity for carcinoembryonic antigen (CEA) by expression of the CEA-specific CAR BW431/26scFv-Fc-CD3ζ (supplementary Fig. 1a). The CAR redirected the activation of engineered T cells in an antigen-restricted fashion, indicated by induced IFN-γ secretion and cytolysis of CEA+ LS174T tumor cells but not of CEA− L540 cells (supplementary Fig. 1b). For comparison, T cells engineered with an anti-CD30 CAR were activated in cytokine secretion and cytolysis by CD30+ CEA− L540 cells but not by CD30− CEA+ LS174T cells. T cells without CAR were not activated by LS174T or L540 cells. The CAR redirected anti-tumor cell attack retained specificity in the presence of added IL-12 as demonstrated by co-incubation of anti-CEA CAR engineered T cells with tumor cells in presence or absence of added IL-12 (supplementary Fig. 1c). While the cytolytic activity towards CEA+ tumor cells was not altered, IFN-γ release was substantially increased by IL-12. T cell populations consisted of about 65% CD8+ and 35% CD4+ T cells after in vitro engineering and amplification. Since adoptive transfer of both T cell subsets is superior in adoptive cell therapy compared to CD8+ T cells only (11) we used the mixture of both T cell subsets in the following analyses.

T cells redirected by a CEA-specific CAR were engineered to express single-chain p40-p35 IL-12 under the control of the (NFAT)₆ responsive element to compel inducible IL-12 (iIL-12) release upon CAR engagement of CEA+ tumor cells. Anti-CEA CAR and iIL-12 modified T cells secreted increased IL-12 levels upon co-incubation with CEA+ tumor cells, compared to incubation with CEA− tumor cells (Fig. 1a). IFN-γ secretion was moreover increased compared to CAR redirected T cells without iIL-12. Engineered T cells showed redirected cytolysis towards CEA+ tumor cells and not towards CEA− tumor cells, demonstrating
preserved specificity of engineered T cells. IL-12 itself did not induce lysis of the respective tumor cells (supplementary Fig. 1c). T cell secreted IL-12 was functional since cell-free culture supernatant from stimulated, CAR and iIL-12 modified T cells induced increase in CD80 and CD86 expression by isolated mouse CD11b+ macrophages, as did supernatant from T cells with engineered constitutive IL-12 expression. Supernatants from CAR T cells without iIL-12 and from unmodified T cells, in contrast, did not induce macrophage activation (Fig. 1b).

We recorded the in vivo cytolytic activity of modified T cells in the NIH-III mouse (LystbgFoxn1nuBtkxid) which lacks T, B and NK cells but not macrophages. CEA+ C15A3 tumor cells were co-injected with engineered T cells at a 1 : 4.5 T cell-to-tumor cell ratio which results in slightly delayed tumor outgrowth compared to non-modified T cells (Fig. 2a,b). Under these conditions, furthermore delay or repression of tumor outgrowth indicates improvement of anti-tumor cell activity. CAR T cells with iIL-12, in contrast to the same number of CAR T cells without IL-12, efficiently prevented tumor outgrowth (Fig. 2c). Improvement in anti-tumor cell activity required CAR signaling since tumor formation by MC38 cells, which are the CEA-negative parental cells of C15A3, was not impaired by anti-CEA CAR T cells with iIL-12 (Fig. 2d). The addition of irradiated C15A3 cells as stimulators, however, resulted in the abrogation of MC38 tumor formation in the presence of anti-CEA CAR T cells with iIL-12 (Fig. 2e). The effect did not occur when anti-CEA CAR T cells lack iIL-12 (Fig. 2f). We conclude that MC38 tumor cells are not recognized by anti-CEA CAR engineered T cells, but are eliminated when engineered T cells are induced to release IL-12. Co-inoculation of tumor cells together with irradiated, non-tumorigenic 293T cells, which were engineered to constitutively secrete IL-12 (cIL-12), repressed tumor outgrowth (Fig. 2g) confirming our conclusion that MC38 tumor cell elimination was mediated by IL-12 and did not require other T cell functions.
We now addressed the anti-tumor activity of engineered T cells towards established tumor lesions and asked, (i) whether CEA$^-$ tumor cells in a mixed, established tumor can be eradicated, and (ii), whether the IL-12 induced response towards CEA$^-$ tumor cells is restricted to that tumor lesion where CAR engagement occurs. CEA$^+$ C15A3 cells were marked with click beetle (CB) luciferase and CEA$^-$ MC38 cells with renilla (R) luciferase for \textit{in vivo} imaging. We established tumors by subcutaneous transplantation of both CEA$^+$ and CEA$^-$ tumor cells in the right flank and CEA$^-$ tumor cells only in the contralateral flank of the same mouse. When tumors reached a volume of about 40 mm$^3$, T cells were systemically applied by a single i.v. injection into the tail vein. Anti-CEA CAR and iIL-12 engineered T cells prevented further growth of both CEA$^+$ and CEA$^-$ tumor cells in the mixed tumor lesion as made visible by bioluminescence imaging of the individual cell populations (Fig. 3a) and quantified by photon recording (Fig. 3b). In contrast, the CEA$^-$ tumor at the contralateral flank of the same mouse continued to grow progressively. For comparison, CEA$^-$ cells expanded progressively in the mixed tumor lesion upon application of T cells with anti-CEA CAR, but without iIL-12. T cells engineered with iIL-12 and a CAR of irrelevant specificity did not affect growth of CEA$^+$ or CEA$^-$ tumors. The application of non-modified T cells did not alter tumor growth in either cell population. Tumor volumes mirror data obtained by \textit{in vivo} imaging (Fig. 3c). Taken together data demonstrate that CEA$^-$ tumor cells in a mixed tumor lesion can be eliminated by engineered T cells and requires both IL-12 release and specific CAR engagement. Data moreover indicated that the effect is locally restricted to that tumor lesion where CAR activation occurs.

Tumors treated with CAR and iIL-12 modified T cells were infiltrated with increased numbers of macrophages, identified by the CD11a$^+$ CD11b$^+$ CD18$^+$ phenotype, compared to tumors treated with CAR T cells without IL-12 (Fig. 4a). Infiltrating macrophages exhibited
an activated phenotype indicated by IL-12 receptor β1 chain expression and increased expression of CD80 and CD86 compared to the very few macrophages found in tumors treated with CAR T cells without iIL-12 (Fig. 4b).

We hypothesized that macrophages that accumulate in iIL-12 treated tumors in mice lacking T, B and NK cells, are involved in the repression of antigen-loss tumors. To explore whether macrophages in treated tumor lesions secrete tumor-repressive cytokines we screened a series of tumor tissue slides for the respective cytokines. Macrophages in iIL-12 CAR T cell treated tumors produce TNF-α; no other TNF-α producing cells were detected in those tumors (Fig. 5a). Tumors treated with CAR T cells without IL-12 as control did not contain substantial numbers of macrophages or TNF-α producing cells.

We asked whether IL-12 activated macrophages can eradicate MC38 cells. Macrophages were in vivo activated by IL-12 and isolated to homogeneity from the peritoneal cavity of treated mice. Co-incubated with MC38 cells in vitro, macrophages eliminated MC38 cells in a dose-dependent fashion; IL-12 activated macrophages showed substantially improved MC38 killing (Fig. 5b) demonstrating potent anti-tumor cell activity of IL-12 activated macrophages.

To demonstrate that macrophages are involved in the process in vivo, macrophages were eliminated in those mice by myeloablative pre-treatment with cyclophosphamide and fludarabine (Fig. 5c). Since the effect is independent of T cell effector functions (cf. Fig. 2g), we co-inoculated MC38 cells together with irradiated 293T cells that constitutively secrete IL-12. MC38 cells established progressively growing tumors in macrophage depleted mice despite IL-12 supplementation whereas MC38 cells were eliminated in non-depleted mice. Supplementation of flow sorted macrophages to myeloablated mice restored the anti-MC38 activity.
tumor cell activity in presence of IL-12. We conclude that macrophages are involved in the IL-12 induced elimination of MC38 tumor cells.

Macrophages in treated tumors produced TNF-α and were the only TNF-α producers in those lesions. MC38 tumor cells were sensitive to TNF-α since recombinant TNF-α added to a culture of proliferating MC38 cells decreased viability in a dose-dependent manner (Fig. 5d). We therefore explored whether MC38 tumor cell elimination occurs in a TNF-α dependent fashion in vivo. Tumors consisting of both CEA+C15A3 cells and luciferase marked CEA-MC38 cells were established in mice by s.c. injection. Mice were treated by i.v. injection of iIL-12 CAR T cells followed by the application of a neutralizing anti-TNF-α antibody or an antibody of irrelevant specificity as control. As summarized in Fig. 5e, MC38 tumors progressed in mice treated with the neutralizing anti-TNF-α antibody whereas MC38 cells ceased amplification in mice treated with a control antibody. We conclude that CEA-MC38 tumor cells were eliminated in a TNF-α dependent fashion upon adoptive therapy with iIL-12 CAR T cells.
DISCUSSION

Constitutive IL-12 expression by adoptively transferred, redirected T cells was recently reported to substantially improve anti-tumor efficacy, i.e., a single dose of $10^4$ IL-12 modified T cells was therapeutically effective against established tumors compared to $2 \times 10^7$ T cells without IL-12 (12). Application of high doses of systemically applied IL-12 does not recapitulate the effect implying that constitutive IL-12 production of engineered T cells may provide therapeutic benefit. In contrast to the study, we here used T cells with inducible IL-12 in order to deliver the cytokine only when adoptively transferred T cells engage cognate antigen. There are a number of advantages of controlled IL-12 supplement in the tumor environment through CAR redirected T cells. First, systemic IL-12 application induces severe toxicity including adverse hematopoietic, intestinal, hepatic and pulmonary effects (13), probably mediated by induction of high IFN-$\gamma$ levels, which prevents systemic application in therapeutically effective levels. We do not expect such toxicity upon CAR controlled locally restricted IL-12 supplementation through engineered T cells. Second, local IL-12 installation into metastatic tumor lesions is mostly not feasible, particular for brain metastases and multiple metastases in inner organs, which requires a vehicle for IL-12 transport. Due to their tissue penetrating capacities, engineered T cells are assumed to be of benefit. Third, once activated engineered T cells continuously produce IL-12 providing constantly high cytokine levels in the targeted organ. IL-12 production, however, ceases when T cells no longer engage the CAR-defined antigen. Forth, inducible IL-12 expression moreover has the advantage to avoid apoptosis of engineered T cells during *ex vivo* amplification, which is a major obstacle when amplifying T cells with constitutive IL-12 expression (14). Fifth, local supplementation with IL-12 recruits and activates innate immune cells that mediate an antigen-independent anti-tumor reaction in the tumor lesion resulting in the elimination of antigen-loss tumor cells.
Due to the synergistic effect of IL-12 and T cell secreted IFN-γ we moreover assume that T cell anti-tumor efficacy is improved compared to that without IL-12.

Previous studies including the most recent report by Zhang and colleagues (14) showed improved efficacy of IL-12 modified T cells towards large established tumors. Whereas those tumors originated from an established cell line with homogenous expression of the targeted antigen we here targeted tumor lesions that consist of both antigen-positive and negative cancer cells mimicking the clinically relevant situation of a mixed tumor with a substantial number of cancer cells lacking the targeted antigen. IL-12 supplementation did not only enhance the CAR-mediated T cell anti-tumor effect but, noteworthy, also initiated a process which results in the elimination of those tumor cells that lack T cell targeted antigen and are otherwise not recognized in such mixed tumors. While CEA- tumor cells in mixed tumors were efficiently eliminated, homogeneous CEA- tumors, however, were not eradicated due to lack of T cell activation through their CAR.

The therapeutic effect of IL-12 was attributed to enhancing cytolytic activity of NK and CD8+ T cells (6) and of stimulating a subset of NKp46+ cells (15). Based on following data we conclude that elimination of tumor cells lacking the cognate T cell antigen is mediated by macrophages. Tumor lesions treated with CAR and iIL-12 engineered T cells were infiltrated with a substantial number of macrophages that were not found in tumors upon T cell therapy without IL-12. Infiltrating macrophages were activated, indicated by IL-12Rβ1 chain and increased CD80/CD86 expression. Macrophage elimination in vivo abrogates MC38 elimination upon IL-12 therapy; add-back of purified macrophages reconstituted the effect. Macrophages produce TNF-α, MC38 tumor cells are sensitive to TNF-α, and blocking TNF-α in vivo abrogated MC38 cell elimination by iIL-12 engineered T cells. We therefore conclude that elimination of CEA- MC38 cells occurred via TNF-α. In the immune competent
host, however, several additional innate subset cells, including NK and NK-T cells (16, 17) are additional targets for iIL-12 to contribute to TNF-α secretion and elimination of antigen-loss tumor cells. IL-12 moreover promotes an anti-tumor response by cooperating with other cytokines of the cytokine network including IFN-γ to stimulate innate immune cell activation. IL-12 moreover counteracts angiogenesis through the induction of IFN-γ inducible genes and by strengthening the lymphocyte-endothelial cell cross talk.

Clinical trials previously explored alternative strategies to deliver IL-12 locally controlled to the tumor lesion, including the transfer of IL-12 gene-transduced tumor cells (18), fibroblasts (19), or dendritic cells (20). IL-12 production at the tumor environment was associated with substantial macrophage infiltration, vessel damage and necrosis (21). Upon intra-tumoral injections, response rates were about 43% - 56% in cutaneous T cell lymphoma, Kaposi’s sarcoma and mycosis fungoides; in other tumor entities, however, the efficacy was minimal highlighting the need to combine IL-12 with other anti-tumor strategies, e.g., IL-12 together with GM-CSF, both cytokines loaded on microspheres for slow release to the environment (22). Compared to those strategies, CAR and iIL-12 modified T cells have the advantage to deliver IL-12 in a controlled fashion preferentially to the targeted tissue and less to other organs. Tumor trapped T cells continuously produce IL-12 as long as T cell activation occurs. Similarly, Epstein-Barr virus (EBV)-specific, cytolytic T cells were engineered to constitutively express single-chain p40-p35 IL-12 in order to deliver IL-12 to the targeted EBV+ Hodgkin’s lymphoma lesion (23). Engineering with a CAR has the advantage over EBV-specific T cells to allow redirecting IL-12 producing T cell towards any tissue as far as a CAR engaging target is available.

We assume that engineered T cells will additionally change the immunosuppressive environment in the tumor lesion to a Th1 response since IL-12 activated T cells secrete
increased amounts of Th1 cytokines and reduced IL-4 and IL-5 levels. On the other hand, IL-
12 contributes to immune repression through induction of IL-10 (ref. 24) which can be
counteracted by blocking IL-10 receptor binding by co-administration of soluble IL-10R or of
a neutralizing IL-10 antibody. The anti-tumor effects of iIL-12 engineered T cells may be
further improved when administered together with other cytokines, such as IL-2 and IL-18
(25), or with tumor cells with costimulatory ligands (18).

Newly discovered IL-12 family members may be alternatives to be inducible expressed by
redirected T cells. IL-23 and IL-27 affect IFN-γ production of NK cells and re-activation of
polarized T cell responses (26). IL-23 moreover sustains inflammatory diseases and thereby
promotes tumor progression (27) making IL-23 a less favorite candidate. To recruit and
activate innate immune cells IL-18 in concert with T cell secreted IFN-γ may be of benefit
(28, 29). In contrast to IL-12, IL-18 promotes both Th1 and Th2 responses and, in synergy
with IL-12, enhances innate immune cell activity and IFN-γ production. NK cells and
macrophages express the corresponding receptors for both IL-12 and IL-18, and produce IFN-
γ in response to those cytokines providing a rationale to engineer T cells with inducible IL-18
in order to eradicate antigen-heterogeneous tumors.
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AUTHOR’S CONTRIBUTION

M.C. performed the experiments in this study, C.K. generated the inducible IL-12 vector, M.C., A.A.H., and H.A. designed the project and wrote the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1

*Engineered T cells with CAR induced IL-12 release.*

(a) T cells were engineered with the anti-CEA CAR without or with inducible IL-12 (iIL-12), respectively. To record redirected T cell activities, T cells with or without (w/o) CEA-specific CAR and iIL-12 expression were co-incubated in increasing numbers (0.6 - 10 x 10³ engineered T cells/well) with CEA⁺ LS174T and CEA⁻ Colo320 tumor cells (2.5 x 10⁴ cells/well). After 48 hrs, IFN-γ and IL-12 in the culture supernatants were determined by ELISA and specific cytotoxicity was monitored by a XTT-based cytotoxicity assay. Data represent the mean ± standard error of the mean of triplicates.

(b) iIL-12 released by engineered T cells activates macrophages. Cell-free supernatants from cocultures (a) were added to cultures of isolated CD11b⁺ macrophages (each 5x10⁴ cells) from C57/bl6 mice. After 36 hrs macrophages were stained with a PerCP-conjugated anti-CD80 mAb and a PE/Cy7-conjugated anti-CD86 mAb and analyzed by flow cytometry. Changes in CD80 and CD86 expression levels were recorded based on the mean fluorescence intensity (MFI). Statistical analyses were performed using the Student’s t test.

Figure 2

*Inducible IL-12 release by engineered T cells prevents tumor formation by CEA⁻ tumor cells not recognized by the CAR.*

T cells were engineered to express the anti-CEA CAR with or without iIL-12. Non-modified T cells (w/o) served as controls. Engineered T cells (2 x 10⁵ cells) were subcutaneously inoculated together with CEA⁺ C15A3 cells (9 x 10⁵ cells) in NIH-III mice (6-7 mice per group) and tumor formation was monitored (a-c). To demonstrate the necessity of IL-12
release in the elimination of those tumor cells not recognized by the CAR, engineered T cells were co-inoculated with tumor forming CEA⁻ MC38 cells in presence or absence of irradiated CEA⁺ C15A3 cells that provide CEA for CAR stimulation (d-f). For comparison, tumor cells were co-inoculated with irradiated 293T cells engineered to constitutively secrete IL-12 (cIL-12) (g). Of note, survival of tumor cells in vitro was not impaired by added IL-12 (supplementary Fig. 1c). Diagrams show individual tumor volumes, bold lines the mean.

Figure 3

CEA⁺ tumor cells are eliminated in those established tumor lesions in which CAR T cells become activated to release IL-12.

Tumors were induced by s.c. transplantation of CEA⁻ MC38 tumor cells in the left flank and of CEA⁺ C15A3 together with CEA⁻ MC38 cells in the right flank (each 1x10⁶ cells/mouse, 6 mice per group). CEA⁺ tumor cells were marked with click beetle (CB) luciferase, CEA⁻ tumor cells with renilla (R) luciferase. T cells were engineered with anti-CEA CAR with or without iIL-12. As controls, T cells were engineered with the CD30-specific CAR and iIL-12 or left unmodified (w/o). When tumors established to a volume of about 40 mm³, engineered T cells (1x10⁶ cells/mouse) were applied by i.v. injection (CD8⁺ : CD4⁺ T cell ratio routinely 2 : 1) (a) Individual tumor cell populations were recorded by in vivo imaging at day 0 and 19 after T cell injection using the Photon Imager device. One out of 6 mice is exemplarily shown. (b) The photon emission of CEA⁻ MC38 cell population at day 19 in left flank tumors (consisting of MC38 cells only) and in right flank tumors (consisting of C15A3 and MC38 cells), respectively, were quantitatively recorded by renilla luciferase imaging. Photon emission intensity in regions of interest was determined as described in Materials and Methods. (c) Volumes of tumors on the right and left flank were determined using a digital caliper.
Figure 4

Activated macrophages accumulate in tumors upon iIL-12 release by engineered T cells.

Tumors treated with anti-CEA CAR T cells with or without iIL-12 were analysed at day 5 for the presence of macrophages by staining for CD11a, CD11b, CD18, and IL-12Rβ1 receptor (a) and CD11b, CD80, CD86, and IL-12Rβ1 (b). The numbers of macrophages per optical microscope field were determined. (c) The levels of CD80 and CD86 expression were determined by recording the fluorescence intensity (FI) per spot using stainings shown in (b). Statistical analyses were performed using the Student’s t test.

Figure 5

Tumor regression upon transfer of iIL-12 and CAR engineered T cells occurs in a TNF-α dependent fashion.

(a) Mixed CEA+/CEA− tumors treated by application of anti-CEA CAR T cells with or without iIL-12 were analysed at day 5 of treatment for the presence of F4/80+ macrophages. Infiltrating macrophages are activated indicated by IL-12Rβ1 expression and TNF-α production. No other TNF-α secreting cells were detected in the sections. (b) IL-12 activated macrophages kill MC38 cells in vitro. Macrophages were activated in C57BL/6-Rag2tm1Cgn/J mice by intraperitoneal injection of irradiated 293T cells with or without constitutive IL-12 secretion and isolated from the peritoneal cavity two days after treatment. Flow sorted F4/80+ macrophages (1 x 10^3 – 6.25 x 10^4) were co-incubated in vitro with MC38 cells (2.5 x 10^4) for 48 h and the viability of MC38 cells was determined. Data represent the mean of triplicates of three mice. (c) Elimination of macrophages abrogates IL-12 initiated repression of CEA− MC38 tumors. F4/80+ macrophages were eliminated from C57BL/6-Rag2tm1Cgn/J mice by myeloablative treatment. Myeloablated mice (6 mice per group) were
subcutaneously co-injected with MC38 tumor cells (1.5 x 10^6 cells per mouse), irradiated 293T cells with constitutive IL-12 expression (10^6 cells per mouse), together with and without isolated F4/80^+ macrophages (2 x 10^5 cells per mouse), which were flow isolated from the peritoneal cavity of IL-12 treated C57BL/6 mice. As additional controls mice without myeloablative pretreatment were co-injected with MC38 tumor cells and irradiated 293T cells with or without IL-12 expression. (d) MC38 tumor cells (2 x 10^4 cells/well) were cultured in presence of increasing concentrations of TNF-α (1 ng/ml – 2 µg/ml) or mouse IgG control protein for 36 h. Viability of tumor cells was determined by a XTT-based viability assay. Data represent the mean of triplicates. (e) Mixed tumors consisting of both C15A3 (CEA^+) and MC38 (CEA^- rLuc^+) tumor cells were induced by s.c. co-injection of tumor cells (10^6 cells each per mouse). When tumors were established, T cells engineered with CEA-specific CAR and iIL-12 expression were injected i.v. into the tail vein at day 0 (2 x 10^6 cells per mouse). Mice were treated with a neutralizing rat anti-mouse TNF-α antibody or as control with an isotype matched IgG antibody of irrelevant specificity (150 µg/mouse) at days -1, 0, +3, +5, +7. Renilla luciferase marked MC38 cells in the mixed tumors were recorded by bioluminescence imaging and data calculated as described in Materials and Methods.
Figure 1

a. LS174T (CEA+) and Colo320 (CEA) cells treated with IL-12, IFN-γ, or anti-CEA CAR effector T cells.

b. CD80 and CD86 expression in cells treated with or without anti-CEA CAR and IL-12.
Figure 2

C15A3 (CEA+)

a) T cells w/o + iIL-12

b) T cells CAR + iIL-12

c) T cells CAR + iIL-12 + irrad. C15A3 (CEA+)

MC38 (CEA-)

d) T cells CAR + iIL-12 + irrad. C15A3 (CEA+)

e) T cells CAR + iIL-12 + irrad. C15A3 (CEA+)

f) T cells CAR + iIL-12 + irrad. C15A3 (CEA+)

g) Irrad. 293T cells ciIL-12

days after tumor inoculation
Figure 3

a

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<tr>
<th></th>
<th>anti-CEA CAR + iIL-12</th>
<th>anti-CEA CAR</th>
<th>anti-CD30 CAR + iIL-12</th>
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</table>

b

![Graph showing MC38 (CEA-) photons/sr (x1,000)](image9)

- MC38 cells in left flank tumor (consisting of MC38 cells (CEA-))
- MC38 cells in right flank tumor (consisting of MC38 cells (CEA-) and C15A3 cells (CEA+))

C

![Graph showing tumor volume (cm^3)](image10)

- left flank: MC38 cells (CEA+)
- right flank: MC38 cells (CEA-) + C15A3 cells (CEA+)
**Figure 4**

(a) **CD11a/CD11b**

- **iiIL-12**
- **w/o IL-12**

- **CD18**
- **IL-12R**
- **Overlay**

**CD11a/b⁺ CD18⁺ IL-12R⁺ cells**

- **p < 0.004**

(b) **CD11b**

- **iiIL-12**
- **w/o IL-12**

- **CD80**
- **CD86**
- **IL-12R**
- **Overlay**

**CD11b⁺ CD80⁺ CD86⁺ IL-12R⁺ cells**

- **p < 0.002**

(c) **CD80**

- **p < 0.005**

- **CD86**

- **p < 0.004**
Figure 5

a
CAR iIL-12 T cell treated tumor

CAR T cell treated tumor

b
![Graph showing viability vs. macrophages]

IL-12
w/o IL-12

0 10 100 1000 10000

macrophages

b
![Graph showing viability vs. macrophages]

IL-12
w/o IL-12

0 20 40 60 80

viability [%]

0 10 100 1000 10000

macrophages

c
![Graph showing tumor volume vs. days]

non-treated mice

myeloablation + IL-12

myeloablation + IL-12 + macrophages

w/o myeloablation + IL-12

w/o myeloablation, w/o IL-12

p < 0.001

c
![Graph showing tumor volume vs. days]

0 5 10 15 20

day

0 500 1000 1500

macrophage depleted mice

isotyped

F4/80

isolated macrophages

myeloablation + IL-12

myeloablation + IL-12 + macrophages

w/o myeloablation + IL-12

w/o myeloablation, w/o IL-12

p < 0.001

c
![Graph showing tumor volume vs. days]

d
![Graph showing viability vs. [ng/ml]]

TNF-α mAb

mlgG

0.1 1 10 100 1000 10000

[ng/ml]

50 75 100

viability [%]

d
![Graph showing viability vs. [ng/ml]]

e
![Graph showing photons/sr vs. days]

anti-TNFα mAb

control IgG mAb

-1 4 9 14 19

day

0 100 200 300 400

photons/sr (x 10^6.0)

-1 4 9 14 19

day

0 100 200 300 400

photons/sr (x 10^6.0)
IL-12 release by engineered T cells expressing chimeric antigen receptors can effectively muster an antigen-independent macrophage response on tumor cells that shut down tumor antigen expression

Markus Chmielewski, Caroline Kopecky, Andreas Hombach, et al.

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