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 interleukin 12 (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 antitumor response, because removing the macrophages abolished the response and restoring them reengaged it. Neutralizing TNF-α also abrogated the elimination of antigen-loss cancer cells, implying this proinflammatory 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 proinflammatory response. *Cancer Res; 71(17); 5697–706. ©2011 AACR.*

Introduction

Adoptive cell therapy with antigen-specific cytotoxic T cells has shown efficacy in fighting malignant diseases in preclinical models and in first clinical trials (1). T cells of desired specificity or tumor infiltrating T cells are isolated, expanded in culture, and reinfused 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 because of 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 deficient in making cancer cells invisible to T-cell recognition. Other mechanisms include an altered cytokine profile and the release of immune repressive cytokines such as TGF-β and interleukin 10 (IL-10), the presence of suppressive cells, and the nonpermissiveness 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

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tumor cells. In those T cells, IL-12 expression is under control of the $NFAT_6$ minimal promoter that initiates IL-12 transcription upon CAR-redirected T-cell activation. The main physiologic producers of IL-12 are phagocytes and dendritic cells in response to pathogens, to T-cell and natural killer (NK) cell signals, and to the components of the inflammatory extracellular matrix (7). IL-12 has a pivotal role in proinflammatory and immunoregulatory functions. Although IL-12 has no direct effect on tumor cells, the antitumor 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 antiangiogenic activities. These privileges explain the considerable efforts to establish IL-12 in tumor therapy. Clinical trials showed some antitumor 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 used the migratory and tissue-penetrating capacities of T cells engineered to target the tumor lesion through their CAR and release iIL-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

Cells from 293T (ATCC CRL-11268), LS174T (ATCC CCL 188), Colo320 (ATCC CCL 220.1), and C15A3 cell lines were provided by Dr. M. Neumaier, Universität Heidelberg-Mannheim, Germany. All cell lines were cultured in RPMI 1640 medium and 10% (v/v) FCS (both from Life Technologies). The anti-human IFN-γ monoclonal antibody (mAb) NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3, the anti-IL-12 p40/p70 mAb C15.6 and its derivatives, and the phycoerythrin (PE)-conjugated anti-mouse IL12Rβ1 mAb 114 were purchased from BD Bioscience. 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 Flour 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. The PerCP-Cy5.5–labeled anti-mouse CD11b mAb M1/70 was purchased from BD Bioscience. The fluorescein isothiocyanate (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, using human $CD3^+$ Micro Beads (Miltenyi Biotec). Murine macrophages from peritoneal cavity were stained by an Alexa Fluor 488–labeled anti-F4/80 mAb A3-1 (Biozol) and isolated by fluorescence-activated cell sorting (FACS) by using a BD FACS Aria III.

CAR modification and activation of T cells

The generation of the retroviral expression cassettes for the carcinoembryonic antigen (CEA)-specific CAR BW431/26scFv-Fc-CD3ζ and the CD30-specific CAR HRS3scFv-Fc-CD3ζ has been previously described in detail (11). To generate the retroviral expression cassette for the pSIN-$(NFAT)_6$-IL-12 vector, the NFAT recognition sequence was amplified from pSIN-$(NFAT)_6$-eGFP vector (12) by PCR, using the following set of primer oligonucleotides (*Bgl*II and *Bam*HI restriction sites are underlined): 5′-GGGCAGATCTAAGCTTGATATCGAATTAGG-3′ (sense) and 5′-CCGAGGATCCAGGAGTTGAGGTTACTGTGA-3′ (antisense). The constitutive cytomegalovirus promoter/enhancer long terminal repeat sequence was replaced by the amplified $NFAT_6$ DNA sequence. In a 2-step transduction procedure, T cells were retrovirally transduced (13) with the iIL-12 expression cassettes and positively selected in the presence of 0.5 mg/mL Geneticin (G418; Sigma-Aldrich) and IL-2 (500 IU/mL) on plates precoated 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, using a PE-conjugated anti-hIgG1 antibody and an FITC-conjugated anti-CD3 mAb (UCHT-1), with a FACS Canto cytofluorometer (BD Bioscience). IFN-γ in the culture supernatant was recorded by ELISA by 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 hours in 96-well plates. The specific cytotoxicity of macrophages was monitored by 2,3-bis[2-methoxy-4-nitro-S-sulfophenynl]H-tetrazolium-5-carboxanilide inner salt (XTT)-based colorimetric assay (Roche Diagnostics).

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 (*Lyst*^{bg}*Foxn1*^{nu} *Btk*^{xid}; Charles River), which is deficient in NK cells, B cells, and T cells. CEA^+ C15A3 tumor cells (9×10^5 cells/mouse) were s.c. coinjected together with engineered T cells (2×10^5 T cells/mouse) into NIH-III mice (6–7 mice per group). Alternatively, tumors were induced by s.c. injection of C15A3 tumor cells and T cells were applied by i.v. injection at day 6. Tumor growth was monitored daily by external measurement with 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) as substrate for the CB and R luciferase, respectively, was intraperitoneally injected. Bioluminescence was recorded by a Photon Imager biolumines-

cence device (Biospace Lab) equipped with the Photo Vision software (BioSpace) for data postprocessing. The exposure time was 300 seconds for all recordings. The threshold of bioluminescence signals was automatically determined with the use of Photo Vision software. Bioluminescence signals were accordingly filtered. Regions of interest (ROI) were defined as regions above threshold and automatically gated by appropriate program tools. There was no manual gating of ROIs to avoid any incoherence. Photon emission intensity (photon/s/sr) was calculated from data of emitted photons from the respective ROIs by using the Photo Vision software.

TNF-α neutralization

Tumors were induced by s.c. coinjection of C15A3 (CEA^+) and CB luciferase–marked MC38 (CEA^-) tumor cells (5×10^5 cells each) 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) at days −1, 0, +3, +5, and +7. The isotype-matched rabbit immunoglobulin G (IgG) antibody RTK2071 of irrelevant specificity (Biolegend) served as control. T cells expressing anti-CEA CAR and iIL-12 were i.v. injected at day 0 (5×10^5 cells per mouse).

Myeloablation and substitution with macrophages

C57BL/6-$Rag2^{tm1Cgn}$/J mice lacking functional T and B cells were treated with myeloablative concentrations of fludarabine phosphate (150 mg/kg body weight; Gry-Pharma) and cyclophosphamide monohydrate (200 mg/kg body weight; Sigma-Aldrich) to deplete NK cells and macrophages. At day 3 after treatment, no NK cells or macrophages were detected in the peripheral blood. The 293T cells engineered to constitutively express IL-12 (10^6 cells) were s.c. injected into pretreated mice together with CEA^- MC38 tumor cells (1.5×10^6 cells) with or without freshly isolated $F4/80^+$ macrophages from C57BL/6 mice (2×10^5 cells). Control groups were coinjections into pretreated mice without macrophages, application of 293T cells without IL-12 expression, and mice without pretreatment.

Immunohistologic 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, and CD86 by the Alexa Flour 647–labeled mAb GL-1. The staining specificity was assayed with the use of isotype-matched control antibodies. A Carl Zeiss Axiovert 400 M microscope was used to analyze the slides.

Results

Peripheral blood T cells were engineered with predefined specificity for CEA by expression of the CEA-specific CAR BW431/26scFv-Fc-CD3ζ (Supplementary Fig. S1A). 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. S1B). 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 antitumor cell attack retained specificity in the presence of added IL-12 as shown by coincubation of anti-CEA CAR-engineered T cells with tumor cells in presence or absence of added IL-12 (Supplementary Fig. S1C). Although the cytolytic activity toward CEA^+ tumor cells was not altered, IFN-γ release was substantially increased by IL-12. T-cell populations consisted of approximately 65% $CD8^+$ and 35% $CD4^+$ T cells after *in vitro* engineering and amplification. Because adoptive transfer of both T-cell subsets is superior in adoptive cell therapy compared with $CD8^+$ T cells only (14), 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 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 coincubation with CEA^+ tumor cells, compared with incubation with CEA^- tumor cells (Fig. 1A). IFN-γ secretion was moreover increased compared with CAR-redirected T cells without iIL-12. Engineered T cells showed redirected cytolysis toward CEA^+ tumor cells and not toward CEA^- tumor cells, showing preserved specificity of engineered T cells. IL-12 itself did not induce lysis of the respective tumor cells (Supplementary Fig. S1C). T-cell–secreted IL-12 was functional because 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 (*Lyst*^{bg}*Foxn1*^{nu}*Btk*^{xid}), which lacks T cells, B cells, and NK cells but not macrophages. CEA^+ C15A3 tumor cells were coinjected with engineered T cells at a 1:4.5 T cell to tumor cell ratio, which results in slightly delayed tumor outgrowth compared with nonmodified T cells (Fig. 2A and B). Under these conditions, furthermore delay or repression of tumor outgrowth indicates improvement of antitumor 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 antitumor cell activity required CAR signaling because tumor formation by MC38 cells, which are the CEA^- 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 lacked iIL-12 (Fig. 2F). We conclude that MC38 tumor cells are not recog-

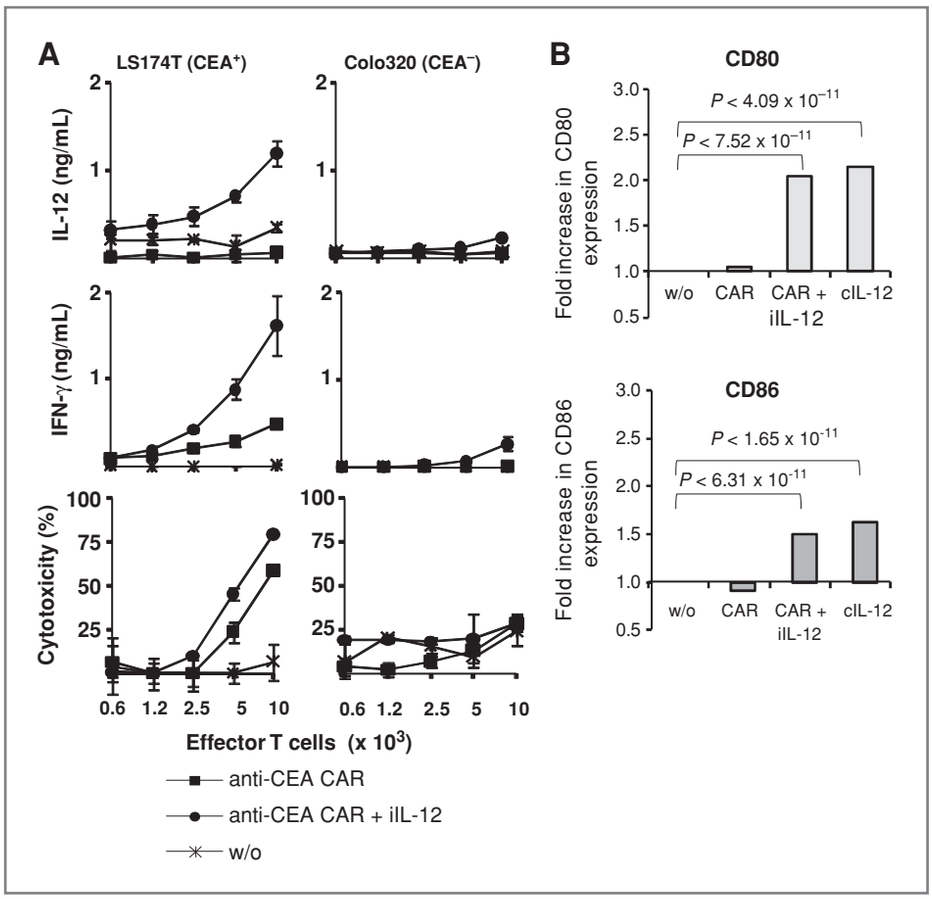

Figure 1. Engineered T cells with CAR-induced IL-12 release. A, T cells were engineered with the anti-CEA CAR without or with iIL-12. To record redirected T-cell activities, T cells with or without (w/o) CEA-specific CAR and iIL-12 expression were coincubated in increasing numbers (0.6×10^3 to 10×10^3 engineered T cells/well) with CEA^+ LS174T and CEA^- Colo320 tumor cells (2.5×10^4 cells/well). After 48 hours, IFN-γ and IL-12 in the culture supernatants were determined by ELISA and specific cytotoxicity was monitored by an XTT-based cytotoxicity assay. Data represent the mean ± SEM 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 (5×10^4 cells each) from C57/bl6 mice. After 36 hours, 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 on the basis of the mean fluorescence intensity. Statistical analyses were carried out by the Student *t* test.

nized by anti-CEA CAR-engineered T cells, but are eliminated when engineered T cells are induced to release IL-12. Coinoculation of tumor cells together with irradiated, nontumorgenic 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 antitumor activity of engineered T cells toward 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 toward CEA^- tumor cells is restricted to the tumor lesion in which CAR engagement occurs. CEA^+ C15A3 cells were marked with CB luciferase and CEA^- MC38 cells with R luciferase for *in vivo* imaging. We established tumors by s.c. 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 approximately 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 nonmodified T cells did not alter tumor growth in either cell population. Tumor volumes mirror data obtained by *in vivo* imaging (Fig. 3C). Taken together, data show 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 the tumor lesion in which 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 with 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 with 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 cells, B cells, and NK cells

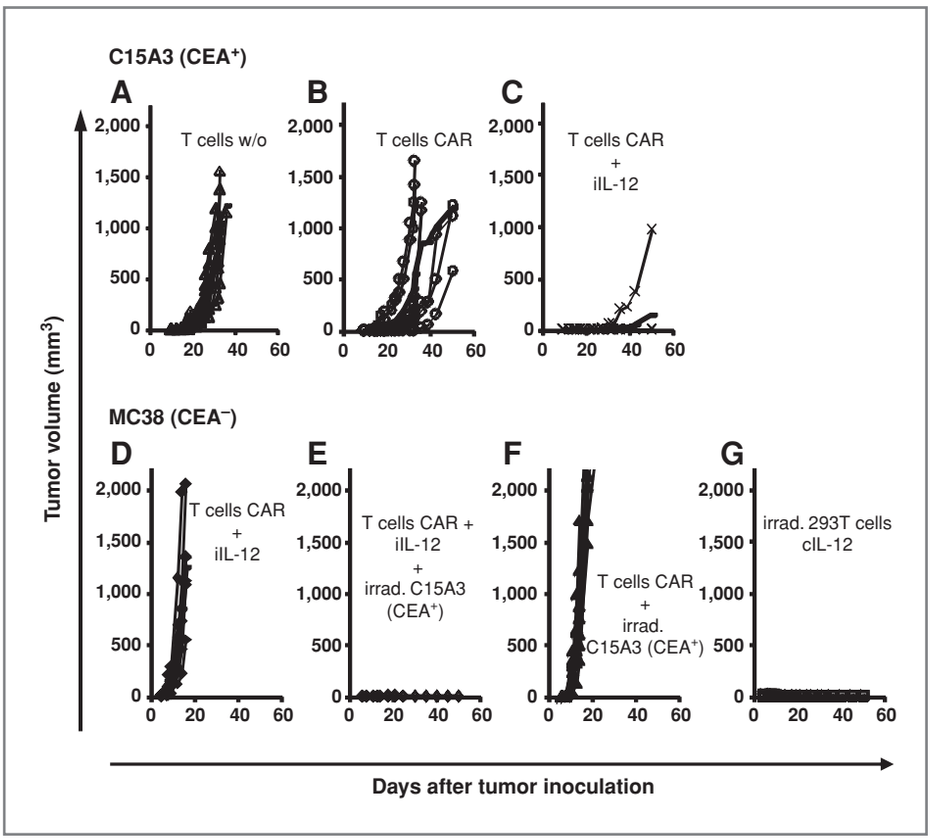

Figure 2. iIL-12 release by engineered T cells prevents tumor formation by CEA$^-$ tumor cells not recognized by the CAR. T cells were engineered to express the anti-CEA CAR with or without iIL-12. Nonmodified T cells (w/o) served as controls. Engineered T cells (2×10^5 cells) were s.c. inoculated together with CEA$^+$ C15A3 cells (9×10^5 cells) in NIH-III mice (6–7 mice per group) and tumor formation was monitored (A–C). To show the necessity of IL-12 release in the elimination of those tumor cells not recognized by the CAR, engineered T cells were coinoculated 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 coinoculated 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. S1C). Diagrams show individual tumor volumes, and bold lines indicate the mean.

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. Coincubated 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), showing potent antitumor cell activity of IL-12–activated macrophages.

To show that macrophages are involved in the process *in vivo*, macrophages were eliminated in those mice by myeloablative pretreatment with cyclophosphamide and fludarabine (Fig. 5C). Because the effect is independent of T-cell effector functions (cf. Fig. 2G), we coinoculated 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 nondepleted mice. Supplementation of flow-sorted macrophages to myeloablated mice restored the anti-MC38 tumor cell activity in the 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 because 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 antitumor efficacy, that is, a single dose of 10^4 IL-12–modified T cells was therapeutically effective against

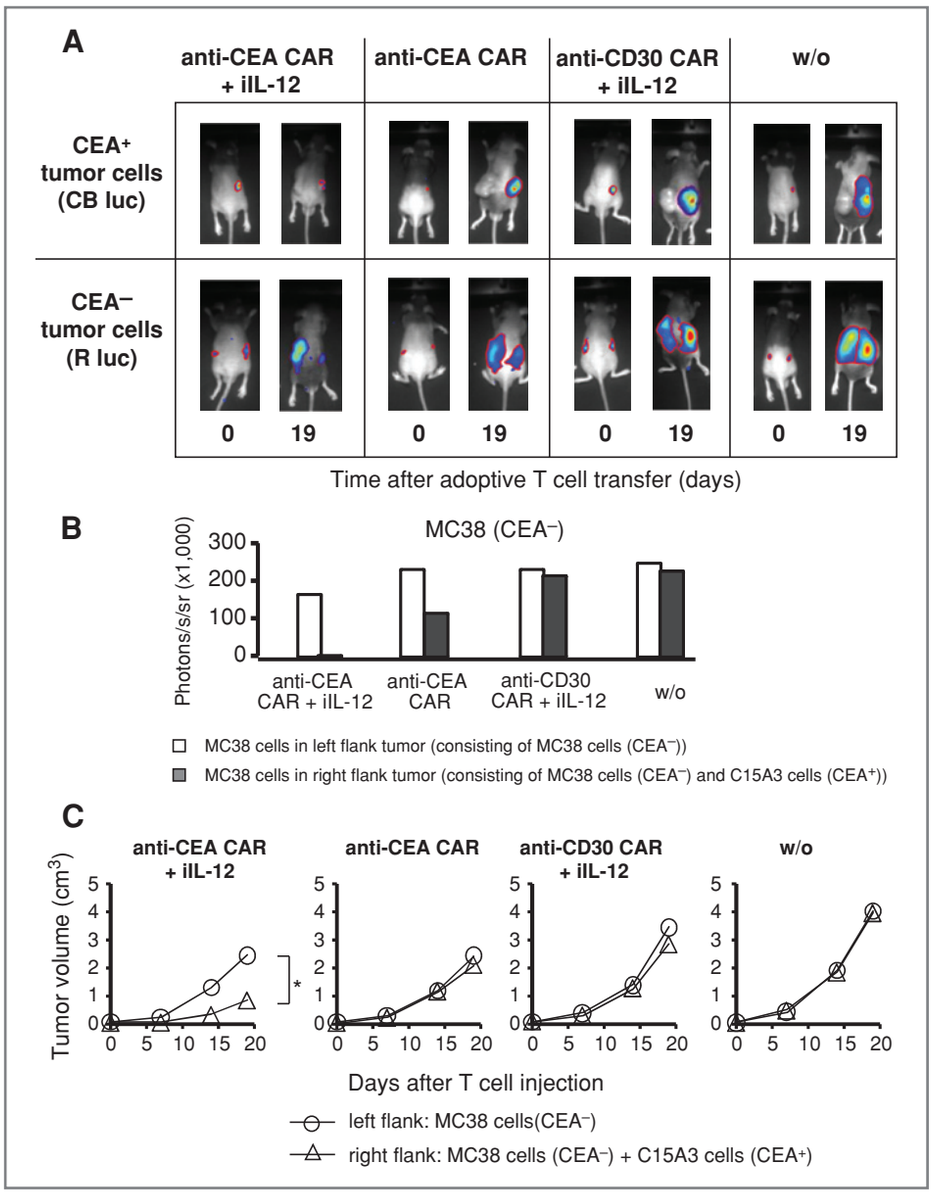

Figure 3. CEA$^-$ tumor cells are eliminated in those established tumor lesions in which CAR T cells become activated to release IL-12. Tumors were induced by s.c. transplantation of CEA$^-$ MC38 tumor cells in the left flank and of CEA$^+$ C15A3 together with CEA$^-$ MC38 cells in the right flank (1×10^6 cells/mouse each, 6 mice per group). CEA$^+$ tumor cells were marked with CB luciferase, CEA$^-$ tumor cells with 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 approximately 40 mm^3, engineered T cells (1×10^6 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 days 0 and 19 after T-cell injection by using the Photon Imager device. One of the 6 mice is shown as an example. 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) was quantitatively recorded by R luciferase imaging. Photon emission intensity in ROIs was determined as described in Materials and Methods. C, volumes of tumors on the right and left flank were determined by a digital caliper.

established tumors compared with 2×10^7 T cells without IL-12 (15). 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, here we used T cells with iIL-12 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 (16), probably mediated by induction of high IFN-γ 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, particularly for brain metastases and multiple metastases in inner organs, which requires a vehicle for IL-12 transport. Because of 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. Fourth, iIL-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 (17). Fifth, local supplementation with IL-12 recruits and activates innate immune cells that mediate an antigen-independent antitumor reaction in the tumor lesion

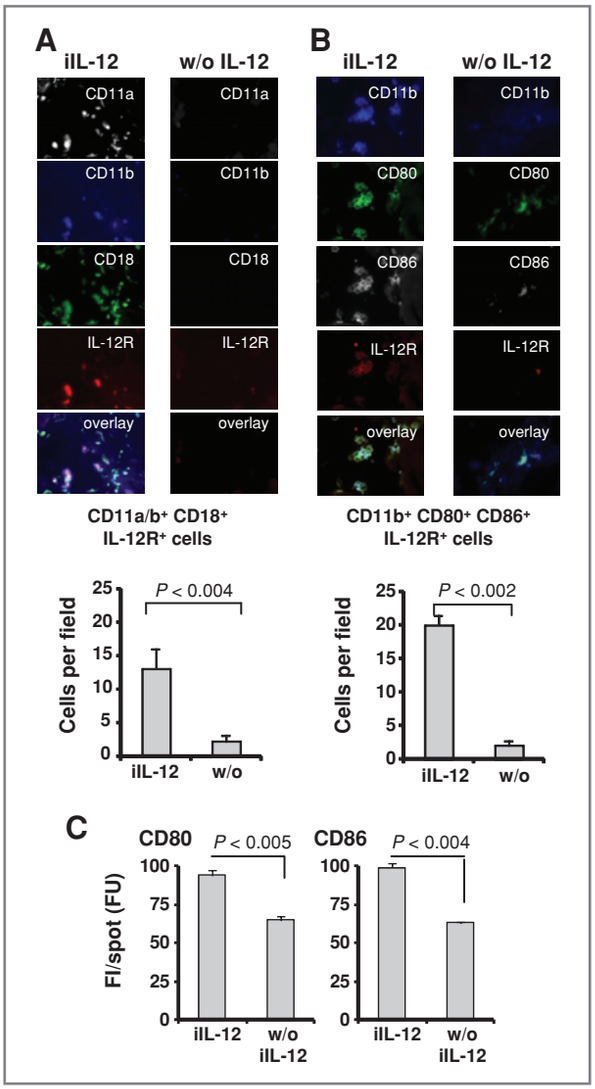

Figure 4. Activated macrophages accumulate in tumors upon iIL-12 release by engineered T cells. Tumors treated with anti-CEA CAR T cells with or without iIL-12 were analyzed at day 5 for the presence of macrophages by staining for CD11a, CD11b, CD18, and IL-12Rβ1 receptor (A) and CD11b, CD80, CD86, and 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 carried out by the Student *t* test.

resulting in the elimination of antigen-loss tumor cells. Because of the synergistic effect of IL-12 and T-cell–secreted IFN-γ we moreover assume that T-cell antitumor efficacy is improved compared with that without IL-12.

Previous studies, including the most recent report by Zhang and colleagues (17), showed improved efficacy of IL-12–modified T cells toward large established tumors. Whereas those tumors originated from an established cell line with homogeneous 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 not only enhanced the CAR-mediated T-cell antitumor effect but, noteworthy, also initiated a process that results in the elimination of those tumor cells that lack T-cell–targeted antigen and are otherwise not recognized in such mixed tumors. Although 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 (18). On the basis of the 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 (19, 20), are additional targets for iIL-12 to contribute to TNF-α secretion and elimination of antigen-loss tumor cells. IL-12 moreover promotes an antitumor 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 (21), fibroblasts (22), or dendritic cells (23). IL-12 production at the tumor environment was associated with substantial macrophage infiltration, vessel damage, and necrosis (24). Upon intratumoral injections, response rates were approximately 43% to 56% in cutaneous T-cell lymphoma, Kaposi sarcoma, and mycosis fungoides; in other tumor entities, however, the efficacy was minimal, highlighting the need to combine IL-12 with other antitumor strategies, for example, IL-12 together with granulocyte macrophage colony-stimulating factor, both cytokines loaded on microspheres for slow release to the environment (25). Compared with 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 to deliver IL-12 to the targeted EBV^+ Hodgkin lymphoma lesion (26). Engineering with a CAR has the

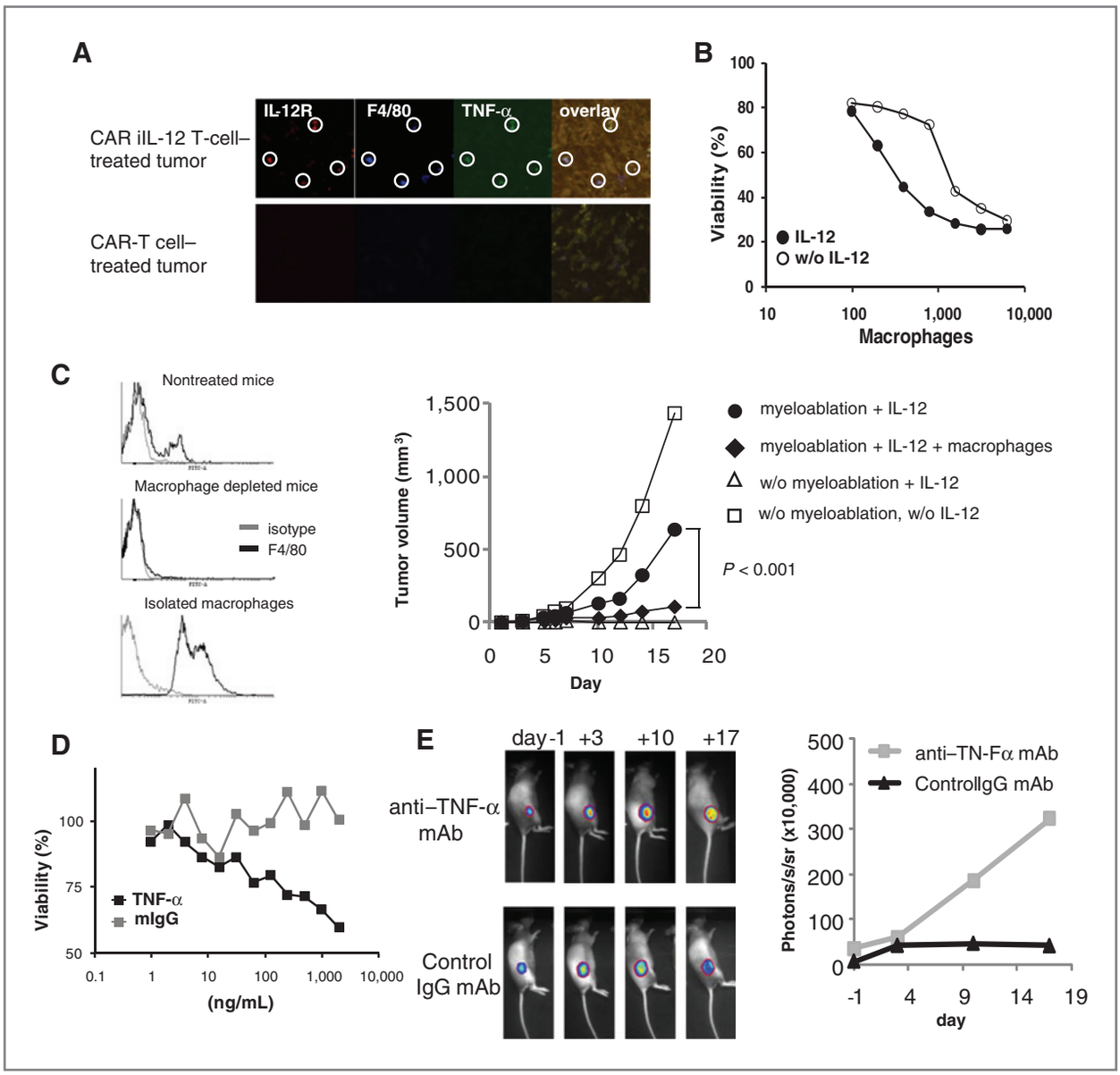

Figure 5. Tumor regression upon transfer of iIL-12–and CAR-engineered T cells occurs in a TNF-α–dependent fashion. A, mixed CEA$^+$/CEA$^-$ tumors treated by application of anti-CEA CAR T cells with or without iIL-12 were analyzed at day 5 of treatment for the presence of F4/80$^+$ macrophages. Infiltrating macrophages are activated, as 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-Rag2^{tm1Cgn}/J mice by intraperitoneal injection of irradiated 293T cells with or without constitutive IL-12 secretion and isolated from the peritoneal cavity 2 days after treatment. Flow-sorted F4/80$^+$ macrophages (1×10^2 to 6.25×10^4) were coincubated *in vitro* with MC38 cells (2.5×10^4) for 48 hours and the viability of MC38 cells was determined. Data represent the mean of triplicates of 3 mice. C, elimination of macrophages abrogates IL-12–initiated repression of CEA$^-$ MC38 tumors. F4/80$^+$ macrophages were eliminated from C57BL/6-Rag2^{tm1Cgn}/J mice by myeloablative treatment. Myeloablated mice (6 mice per group) were s.c. coinjected with MC38 tumor cells (1.5×10^6 cells per mouse), irradiated 293T cells with constitutive IL-12 expression (10^6 cells per mouse), together with and without isolated F4/80$^+$ macrophages (2×10^5 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 coinjected with MC38 tumor cells and irradiated 293T cells with or without IL-12 expression. D, MC38 tumor cells (2×10^4 cells/well) were cultured in presence of increasing concentrations of TNF-α (1 ng/mL to 2 μg/mL) or mouse IgG control protein for 36 hours. Viability of tumor cells was determined by an 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. coinjection 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 (2×10^6 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, and +7. R luciferase–marked MC38 cells in the mixed tumors were recorded by bioluminescence imaging and data calculated as described in Materials and Methods.

advantage over EBV-specific T cells to allow redirecting IL-12–producing T cells toward 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 because IL-12–activated T cells secrete increased amounts of Th1 cytokines and reduced IL-4 and IL-5 levels. On the contrary, IL-12 contributes to immune repression through induction of IL-10 (27), which can be counteracted by blocking IL-10 receptor binding by coadministration of soluble IL-10R or of a neutralizing IL-10 antibody. The antitumor effects of iIL-12–engineered T cells may be further improved when administered together with other cytokines, such as IL-2 and IL-18 (28), or with tumor cells with costimulatory ligands (21).

Newly discovered IL-12 family members may be alternatives to be inducibly expressed by redirected T cells. IL-23 and IL-27 affect IFN-γ production of NK cells and reactivation of polarized T-cell responses (29). IL-23 moreover sustains inflammatory diseases and thereby promotes tumor progression (30), 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 (31, 32). 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 iIL-18 to eradicate antigen-heterogeneous tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

M. Chmielewski carried out the experiments in this study; C. Kopecky generated the inducible IL-12 vector; M. Chmielewski, A.A. Hombach, and H. Abken designed the project and wrote the manuscript.

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