Published OnlineFirst November 14, 2010; DOI: 10.1158/1078-0432.CCR-10-1340

Cancer Therapy: Preclinical

Clinical Cancer Research

Eradication of Medullary Multiple Myeloma by CD4$^+$ Cytotoxic Human T Lymphocytes Directed at a Single Minor Histocompatibility Antigen

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Abstract

Purpose: The essential role of CD4$^+$ T cells as helpers of anticancer immunity is indisputable. Little is known, however, about their capacity to serve as effector cells in cancer treatment. Therefore, we explored the efficacy of immunotherapy with sole CD4$^+$ cytotoxic human T cells directed at a hematopoietic-restricted minor histocompatibility antigen (mHag).

Experimental Design: In macrophage-depleted Rag2$^{-/-}\gamma c^{-/-}$ mice, which were also devoid of T, B, and natural killer cells, mHag-specific native T cells or tetanus toxoid (TT)-specific T cells transduced with the mHag-specific T-cell receptor (TCR) were injected to treat full-blown mHag$^+$ human multiple myeloma tumors.

Results: mHag-specific antitumor responses were achieved after injection of native or mHag-TCR-transduced T cells. Although the therapy completely eradicated the primary tumors in the bone marrow, it failed to control extramedullary relapses, even after repeated T-cell injections. Detailed analyses ruled out mHag or MHC downregulation as mechanisms of extramedullary tumor escape. Impaired T-cell survival *in vivo* or defective homing to the tumor site were also ruled out as mechanisms behind extramedullary relapses, because injections of TT-loaded antigen presenting cells could facilitate homing of long-term surviving T cells to s.c. tumor sites. Moreover, intratumoral treatment of extramedullary tumors with 3AB11 was also ineffective.

Conclusions: Taken together, these results for the first time show the feasibility of immunotherapy of primary bone marrow tumors with sole CD4$^+$ human T cells directed to a tumor-associated mHag. Extramedullary relapses, probably due to microenvironment-dependent inhibitory mechanisms, remain a challenging issue towards effective cellular immunotherapy of hematologic malignancies. *Clin Cancer Res; 16(22); 5481–8. ©2010 AACR.*

The immune system is armed with various cellular tools to combat cancer. Over the past decades, the therapeutic potency of these tools was shown not only by the successful treatment of experimental animal tumors with adoptively transferred T cells, but also by the induction of long-term remissions in leukemia patients after treatment with allogeneic stem cell transplantation and donor lymphocyte infusions (DLI; ref. 1). To date, it is evident that both CD8$^+$ and CD4$^+$ T cells are essential components of antitumor immunity. In the current view, CD8$^+$ T cells are considered the main effector cells of anticancer responses, whereas a helper function is attributed to CD4$^+$ T cells (2–6). Even in studies in which effective antitumor responses have been achieved with CD4$^+$ T cells in the absence of CD8$^+$ T cells, these responses were reported to depend on other immune cells as macrophages or natural killer (NK) cells (7–11). Nonetheless, we and others have shown that several Th1-like CD4$^+$ T cells possess cytotoxic capacity against murine and human tumor cells (12–16). The cytotoxic antitumor activities can even be transferred into recall antigen [tetanus toxoid (TT)]-specific CD4$^+$ T cells by the well-known T-cell receptor (TCR) transfer approach (12). These studies suggest that next to their helper functions, CD4$^+$ T cells may also serve as effector T cells in human cancer immunity for direct killing of tumor cells. At the moment, the need to estimate the *in vivo* impact of CD4$^+$ T cells as sole effector cells in the treatment of hematologic malignancies becomes increasingly urgent

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-10-1340

Published OnlineFirst November 14, 2010; DOI: 10.1158/1078-0432.CCR-10-1340

Translational Relevance

CD4$^+$ T cells are essential as helper T cells in anticancer immunity. Little is known, however, of whether they also serve as effector cells in cancer treatment. We therefore explored the feasibility of cancer treatment with sole CD4$^+$ T cells in a well-established humanized murine model. We show that multiple myeloma tumors primarily progressing in the bone marrow can be effectively eradicated by the injection of a cytotoxic CD4$^+$ human T-cell clone directed at a single minor histocompatibility antigen. The therapy, however, was not sufficient to control extramedullary relapses. Similar results were achieved by injection of recall antigen (tetanus toxoid)-specific CD4$^+$ human T cells that were genetically modified to express the mHag-specific T-cell receptor. Our results for the first time show the sole effector function of CD4$^+$ human T cells against bone marrow tumors in cancer therapy. Extramedullary relapses, however, remain a challenging issue towards effective cellular immunotherapy of hematologic malignancies with sole CD4$^+$ T cells.

because recently we and others have identified a series of new and potentially therapeutic minor histocompatibility antigens (mHag) recognized by CD4$^+$ T cells (14, 15). Furthermore, the *in vivo* antitumor capacity of TCR-modified CD4$^+$ human T cells has never been explored. Therefore, we addressed the feasibility and efficacy of adoptive immunotherapy with sole CD4$^+$ mHag-specific native or TCR-redirected T cells.

To this end we used a bioluminescence imaging (BLI)-based xenografted graft-versus-myeloma model, recently established in immune deficient Rag2$^{-/-}\gamma c^{-/-}$ mice that lack T, B, and NK cells. mHag$^+$ human multiple myeloma tumor inoculated in these mice grew mainly in the bone marrow. After the establishment of progressively expanding medullary tumors, mice were depleted of macrophages and treated with native mHag-specific T cells or mHag-TCR-transduced TT-specific CD4$^+$ T cells. The sole CD4$^+$ T-cell therapy resulted in strong antigen-specific eradication of tumors in the bone marrow, showing for the first time a direct cytotoxic effect of adoptive immunotherapy with native or TCR-redirected dual-specific CD4$^+$ human T cells. The CD4$^+$ T-cell therapy, however, failed to control extramedullary relapses, probably due to deficient infiltration into tumor at extramedullary sites or involvement of tumor microenvironment–dependent T-cell inhibitory mechanisms.

Materials and Methods

Cells

The human *HLA-DPB1*0401*-restricted mHag-specific CD4$^+$ T-cell clone 3AB11 and TT-specific CD4$^+$ T-cell clones were previously described in detail (12, 17). Clone 3AB11 recognizes a yet unknown but potentially therapeutic mHag with hematopoietic-restricted tissue distribution (17). The oligoclonal TT-specific cell line CTL$_{TT}$ was established by mixing four TT-specific CD4$^+$ T-cell clones (N_{TT}3AC6, N_{TT}3AG10, N_{TT}1A3, N_{TT}1E10) in equal proportions. All T cells were expanded using a feeder cell-cytokine mixture as described (12). Luciferase-transduced human multiple myeloma cell line UM9-luc-eGFP was described elsewhere (18). UM9-luc-eGFP and EBV-LCL cells were cultured in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Integro) and antibiotics. All cell lines were authenticated by means of human leukocyte antigen-type and/or surface expression of appropriate receptors within the last six months of research.

Retroviral vectors and transduction of T cells

The retroviral pMX vectors TCRα-IRES-ΔNGF-R and TCRβ-IRES-eGFP, carrying the TCRα and TCRβ chains of clone 3AB11, were described previously (12). The generation of retroviral supernatants, the retroviral transductions of TT-specific T cells, and the Fluorescent Activated Cell Sorter (FACS) sorting (BD) based on eGFP and ΔNGF-R expression were also described (12).

Mice

Rag2$^{-/-}\gamma c^{-/-}$ mice were bred and housed Specified-Pathogen-Free at the Central Animal Facility of the University of Utrecht (19). All animal experiments were conducted according to the Dutch Law on Animal Experiments with permission from the local Ethics Committee for Animal Experimentation.

Transplantation and *in vivo* monitoring of tumor cells

Female mice at 9 to 14 weeks of age received 20 × 10^6 UM9-luc-eGFP cells via the tail vein one day after sublethal irradiation (350 cGy). Tumor growth was quantitatively monitored after i.p. injection of beetle luciferin (2.5 mg; Promega) by either Biospace (Biospace Lab) or Roper (Roper Scientific) BLI systems (18). Arbitrary photon counts of both imaging systems were normalized for comparison of different experiments. In untreated mice, the UM9-luc-eGFP cells grow as a typical multiple myeloma with early-phase growth in bone marrow followed by some metastatic growth at extramedullary foci from 8 weeks on.

Adoptive transfer and monitoring of T cells

At different levels of tumor intensity, mice were macrophage depleted by i.v. injection of fresh 2-chloromethyl biphosphonate (CL2MDP) liposomes as described previously (20). One, three, and six days later the mice were i.v. injected with CD4$^+$ mHag-specific T-cell clone 3AB11, TCR-transduced CTL$_{TT}$ (CTL$_{TT}$-TCR), or the parental control cell line CTL$_{TT}$. *In vivo* monitoring of CTL$_{TT}$-TCR in the mice was carried out by fluorescence imaging (Biospace Lab) up to seven days following i.p. injection with 50 μg α-huCD4-ALEXA-700 (ITK). Two days prior to antibody injection some mice were boosted via injection of TT-loaded (7.5 LF/mL for 48 hours; NVI)

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mHag$^-$ EBV-LCL cells (15×10^6 i.v., 2×10^6 s.c.). Flow cytometry of single-cell suspensions from murine spleens was done using a FACS Calibur after staining with specific conjugated antibodies (BD).

Intratumoral treatment of extramedullary tumors

Rag2$^{-/-}\gamma c^{-/-}$ mice were s.c. inoculated with 9×10^6 UM9-luc-eGFP cells at one flank. Three weeks after inoculation, tumors were treated by intratumoral injections of CD4$^+$ mHag-specific T-cell clone 3AB11 or TT-specific T cells (12×10^6 T cells per tumor; $n = 5$). Separate tumors were also injected with PBS ($n = 5$) as no-treatment control. Tumor progression was monitored by BLI.

Cytotoxicity assay

UM9-luc-eGFP–derived tumors were dissected from various foci of sacrificed mice. Single-cell suspensions of these tumors were then used as target cells in *ex vivo* luciferase-based cytotoxicity assays in white opaque flat bottom 96-well plates (Costar). Effector CD4$^+$ T cells were added at different effector to target (E:T) ratios in the presence of 125 μg/mL beetle luciferin (Promega). At 26 and 48 hours of culture, the light signal emitted from surviving UM9-luc-eGFP cells was measured using a luminometer (Molecular Devices). The percentage lysis was calculated relative to medium control as described (13, 21). Tumor cells from bone marrow could not be subjected to cytotoxicity assays as they displayed poor viability already after one hour of *ex vivo* culture.

Statistical analyses

Unpaired two-tailed Student's *t* tests were used to analyze differences between groups (GraphPad Prism software). *P* values <0.05 were considered significant.

Results

In vivo multiple myeloma reduction by a native CD4$^+$ human T-cell clone directed at a single mHag

In previous studies, we had shown the *in vitro* cytotoxic activity of the mHag-specific Th1-like CD4$^+$ human T-cell clone 3AB11 against the mHag$^+$ human multiple myeloma cell line UM9 (12). To determine its *in vivo* therapeutic potential, we administered this cytotoxic CD4$^+$ T-cell clone into immunodeficient Rag2$^{-/-}\gamma c^{-/-}$ mice, bearing BLI-detectable UM9-luc-eGFP tumors. Three consecutive injections of 3AB11 cells rapidly reduced the tumor load below detection levels, showing for the first time the achievement of a direct antimyeloma response by adoptive transfer of CD4$^+$ human T cells (Fig. 1A and B). No tumor reduction was detected after administration of the control TT-specific T-cell line (CTL_{TT}), showing the antigen specificity of the antitumor effect (Fig. 1A). Treatment of a similar tumor load with 6-fold lower 3AB11 doses or a 3-fold higher tumor load with similar T-cell doses was not effective (data not shown), illustrating the importance of an optimal T-cell dose to tumor load ratio for a successful treatment outcome.

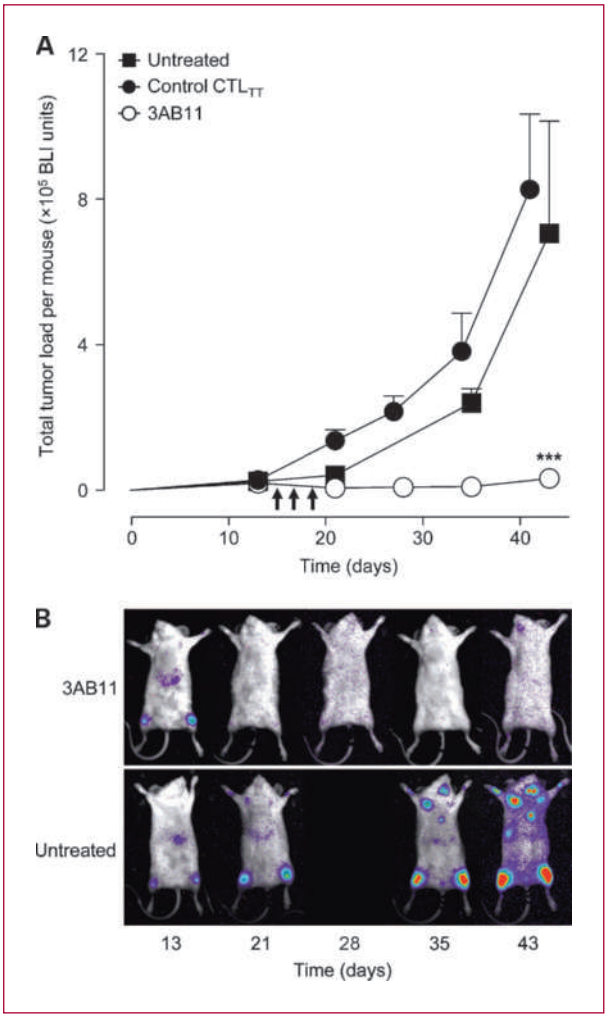

Fig. 1. *In vivo* antitumor effects of a native CD4$^+$ T-cell clone 3AB11 directed at a single mHag. A, UM9-luc-eGFP tumors were established in the bone marrow of Rag2$^{-/-}\gamma c^{-/-}$ mice by sublethal irradiation followed by i.v. injection of 20×10^6 tumor cells. Mice were depleted of macrophages using CL2MDP liposomes one day prior to treatment, consisting of three consecutive i.v. injections (arrows) of 30 to 40×10^6 3AB11 cells/mouse ($n = 5$; ○). As controls, mice were treated with similar injections of CTL_{TT} ($n = 4$; ●) or not treated ($n = 4$; ■). Curves, growth of UM9-luc-eGFP in the mice, measured by BLI of the ventral side (arbitrary units of the normalized photon emission counts). Arrows, T-cell injections. Error bars, SE. At day 43, the difference compared with the untreated group was analyzed using a *t* test (***, $P < 0.001$). B, bioluminescence overlay pictures showing tumor outgrowth over time in representative mice after 3AB11 treatment (top) or no treatment (bottom) between day 13 and day 43.

In vivo antitumor effects of dual antigen-specific TCR-transduced CD4$^+$ T cells

An attractive way to generate sufficient number of antigen-specific T cells for adoptive T-cell therapy is the transfer of antigen-specific TCR into other T cells (22). We had previously shown that this approach is also highly suitable to transfer mHag-specific cytotoxic functions of clone 3AB11 into readily expandable recall antigen (TT)-specific CD4$^+$ T cells (12). To investigate the *in vivo*

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antitumor activity of these dual-specific CD4$^+$ human T cells, we transduced a TT-specific oligoclonal T-cell line (CTL_{TT}) with the TCR of 3AB11. The TCR-transduced cell line, designated as CTL_{TT}-TCR, displayed mHag-specific cytotoxic activity against UM9-luc-eGFP *in vitro* (Fig. 2A and B). Adoptive transfer of CTL_{TT}-TCR into mice carrying established UM9-luc-eGFP tumors induced, similar to parental 3AB11, significant mHag-specific reduction of the multiple myeloma (Fig. 2C). This illustrated the feasibility of establishing effective *in vivo* antimyeloma immunity not only by native mHag-specific T cells but also by TT-specific T cells transduced with the mHag-specific TCR.

Bone marrow restriction of CD4$^+$ T cell–mediated antitumor effects

Clinical treatment of multiple myeloma by DLI is often complicated by extramedullary relapses (23–26). Similar to this clinical scenario, successful treatment with native mHag-specific CD4$^+$ T cells in our model was compromised with progressive outgrowth of multiple myeloma, which seemed to be predominantly located outside the bone marrow (Fig. 3A). Indeed, locus-specific quantification of the BLI data showed that the vast majority of original tumor loci in the bone marrow remained myeloma-free for at least five weeks, whereas extramedullary tumors were progressive (Fig. 3B). Dissection of sacrificed mice revealed that such tumors progressed mainly in ovaries and at s.c. sites (Fig. 3C). Because these results could reflect a (therapy-induced) resistance toward T cell–mediated cytotoxicity, we dissected extramedullary tumors from treated and untreated mice and used their single-cell suspensions as targets for 3AB11. All extramedullary tumor cells derived from either treated or untreated mice were efficiently killed by 3AB11 (Fig. 3D), ruling out the possibility of a therapy-induced resistance of extramedullary tumors via antigen loss or MHC downregulation.

In vivo persistence of dual antigen–specific T cells

An alternative explanation for the extramedullary relapses could be the limited *in vivo* persistence of injected T cells, a well-known caveat of current adoptive T-cell transfer strategies. Supporting this possibility, analyzing spleens of mice treated with dual antigen–specific CD4$^+$ T cells could show the presence of CD4$^+$ human T cells at day 4 but not at day 11 after T-cell injections (Fig. 4A) using FACS-based analyses. In similar assays, we also failed to detect human T cells in other main organs at this time point (data not shown). Nonetheless, because these results could not definitely exclude the possibility that a T-cell population was still persisting in the mice, we attempted to restimulate and visualize the *in vivo* persisting cells by i.v. and s.c. injection of TT-loaded EBV-transformed B cells 29 days after the initial administration of dual antigen (mHag-TT)-specific CTL_{TT}-TCR cells. Using *in vivo* fluorescence imaging, a technique sensitive enough for the tracking of α-huCD4-ALEXA-700–labeled T cells at s.c. sites in a qualitative way (Supplementary Fig. S1), we detected the dual-specific T cells at the sites of s.c. TT-loaded EBV-LCL injection (Fig. 4B, left). There was no CD4$^+$ T-cell accumulation around unloaded mHag$^-$ EBV-LCL cells, which were s.c. injected in a separate mouse as negative control (Fig. 4B, right). In another experiment, we injected a mouse at different s.c. loci with TT-loaded or unloaded EBV-LCL cells and UM9 cells at day 43 after initial injection of CTL_{TT}-TCR. Also in this mouse we observed a clear antigen-specific accumulation of CD4$^+$ T-cell signal at the s.c. sites where TT-loaded EBV-LCL cells or UM9 cells were injected but not at the sites of unloaded EBV-LCL cells (Fig. 4C). All together these assays indicated that (*a*) originally injected dual antigen–specific T cells could persist long term *in vivo* in Rag2$^{-/-}$γc$^{-/-}$ mice, and (*b*) these cells could migrate to s.c. extramedullary tissue in an antigen-specific manner. Nonetheless, in these xenografted mice

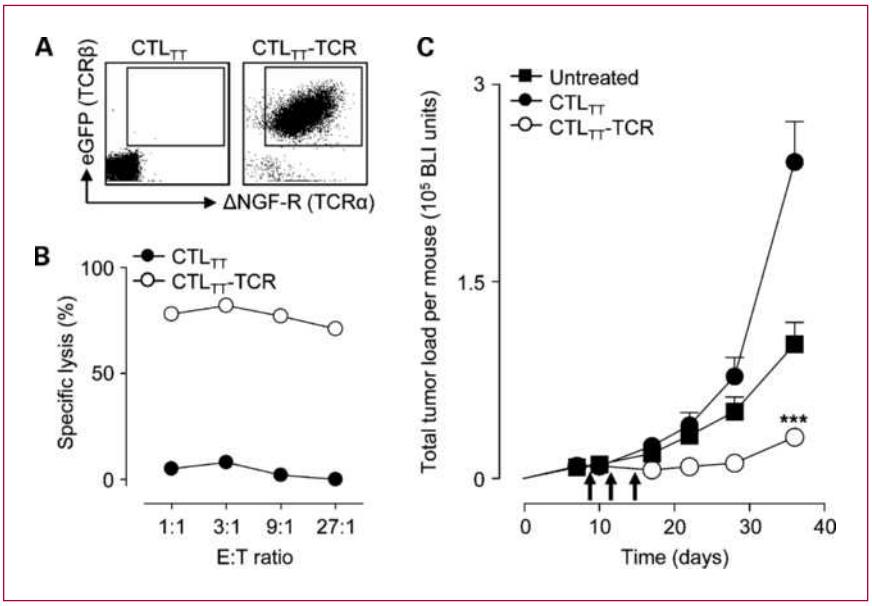

Fig. 2. *In vitro* and *in vivo* antitumor effects of dual TT- and mHag-specific TCR-transduced CD4$^+$ T cells. A, eGFP (representative for TCRβ) and ΔNGF-R (TCRα) expression on the untransduced TT-specific cells CTL_{TT} (left) and on TCR-transduced cells after FACS sorting (CTL_{TT}-TCR; right). B, specific lysis of UM9-luc-eGFP by CTL_{TT} (●) or CTL_{TT}-TCR (○) at indicated effector to target (E:T) ratios after coincubation for 26 hours. Results are the mean percentage lysis. Error bars, SE of triplicate wells. Similar results were obtained in two independent assays. C, Rag2$^{-/-}$γc$^{-/-}$ mice with established UM9-luc-eGFP tumors in bone marrow were treated with 30 to 40 × 10^6 CTL_{TT} ($n = 5$; ●), CTL_{TT}-TCR ($n = 6$; ○), or not treated ($n = 5$; ■) one day after CL2MDP liposome pretreatment. Arrows, days of i.v. T-cell injections. Mean and SE of tumor photon emission are shown per group. The 3AB11-treated group was statistically compared with untreated group at day 36 (***, $P < 0.001$).

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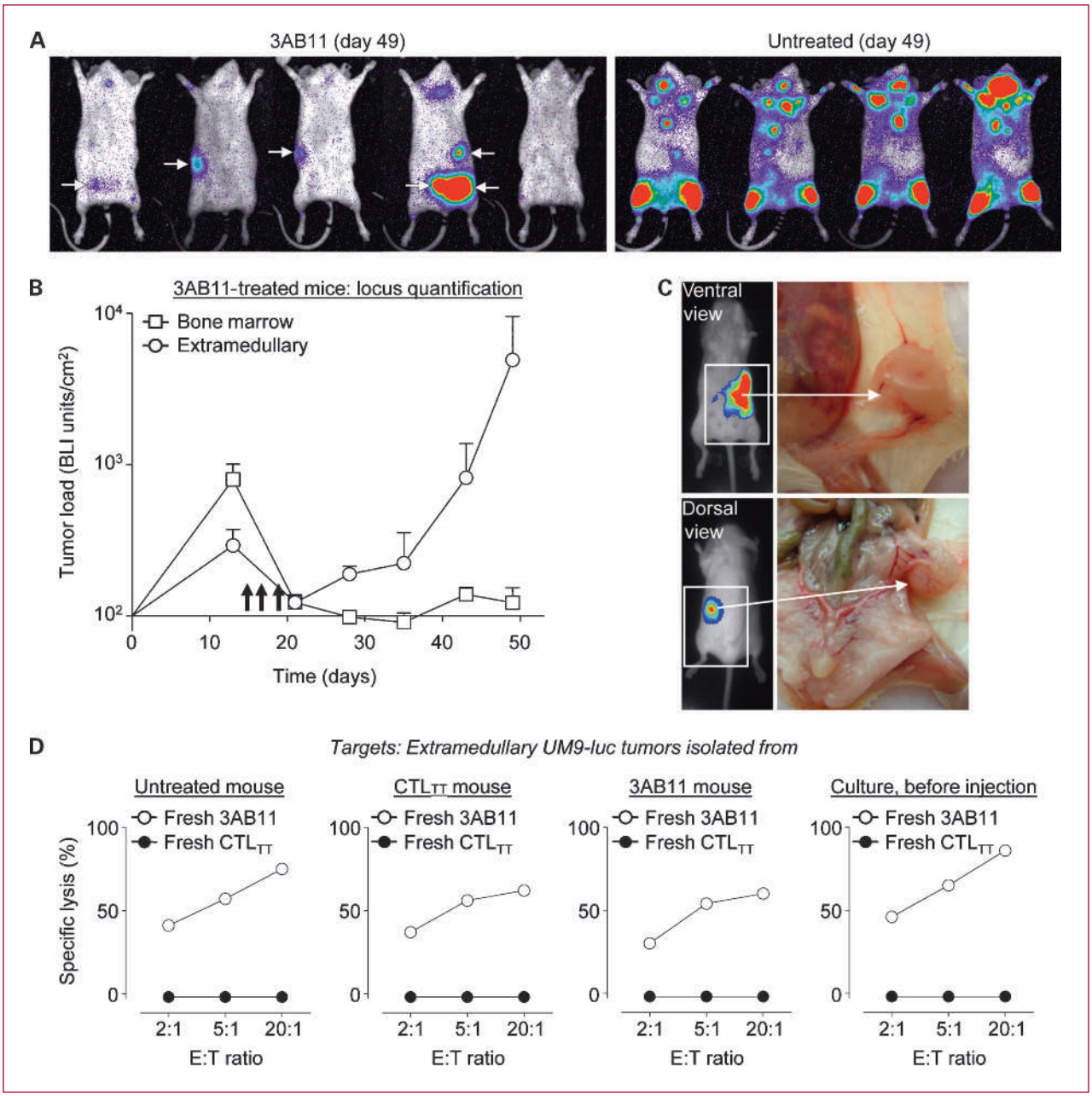

Fig. 3. Extramedullary tumor outgrowth is not resistant to T cell–mediated kill in *ex vivo* analyses. A, tumor load of 3AB11-treated (top) or untreated (bottom) mice at day 49 after injection of UM9-luc-eGFP. Note the outgrowth of extramedullary tumor in treated mice (arrows), whereas the original tumor sites in the bone marrow remain tumor-free. B, locus-specific photon emission was quantified for extramedullary sites (○) and for bone marrow (□) in mice treated with 3AB11 at days indicated by arrows. C, 84 days after T-cell treatment, mice were dissected to visualize extramedullary tumor burdens at luciferase-positive locations. Pictures are representative examples for s.c. (top) and ovary (bottom) tumor relapses, present in respectively 40% and 80% of treated mice. D, single-cell suspensions of UM9-luc-eGFP extramedullary tumors were derived from different treatment groups 84 days after treatment, and used *ex vivo* as targets for fresh 3AB11 cells in a luciferase-based killing assay. Results are the mean lysis percentage of triplicate wells after 48 hours. Error bars, SE. Similar results were obtained in two independent assays.

extramedullary tumors could not be controlled even by additional injections of mHag-specific T cells ~22 days after primary treatment (Fig. 4D). Finally, to investigate failure to eradicate extramedullary sites in the absence of homing-related issues, s.c. inoculated UM9 tumors were treated by intratumoral injections of 3AB11. As shown in Fig. 4E, injection of 3AB11 into the tumor had no significant effect on the growth, indicating that the extramedullary tumors remained resistant to T-cell therapy even after bypassing the homing-related issues. These results strongly

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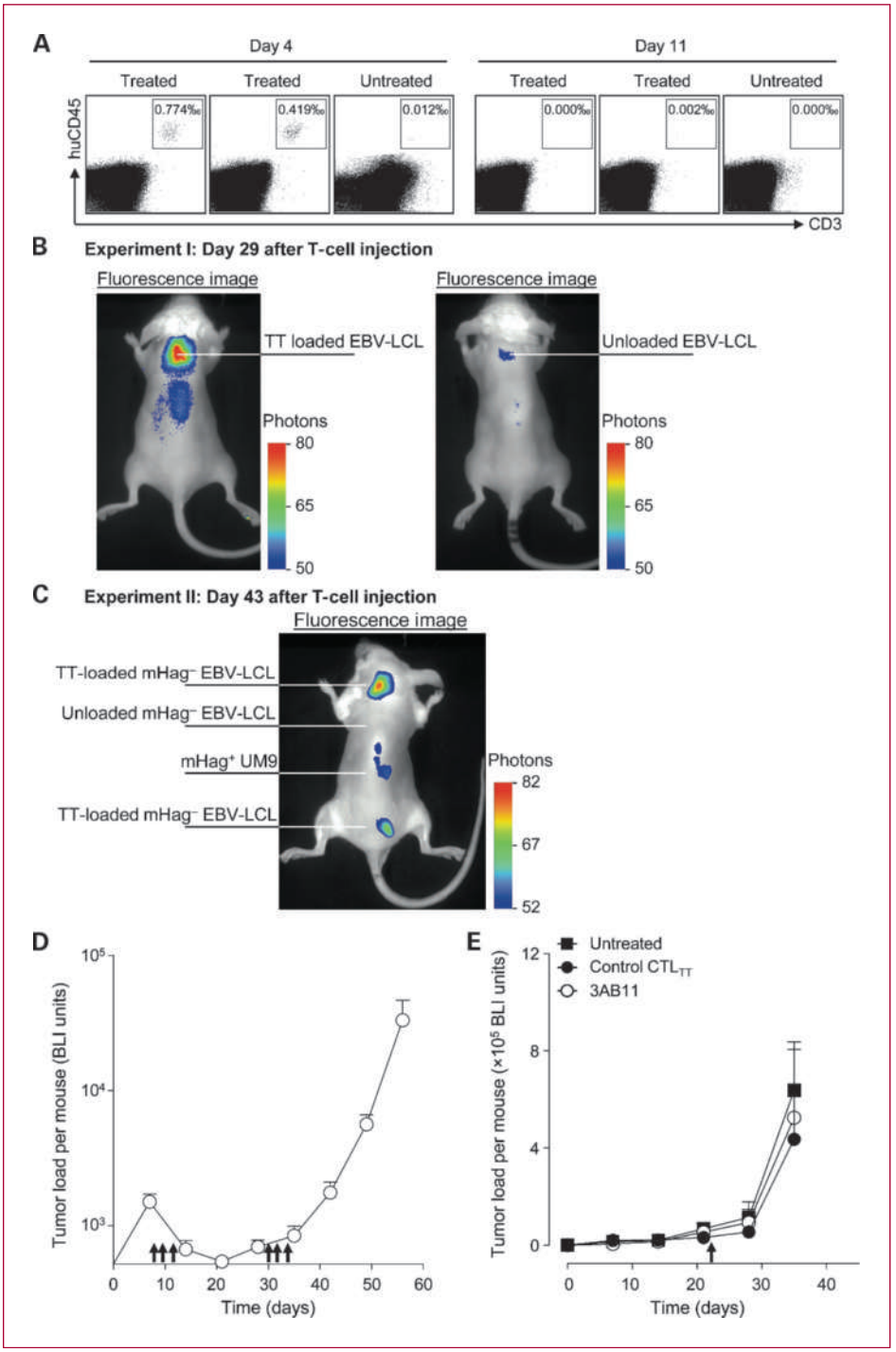

Fig. 4. *In vivo* survival of CD4$^+$ human T cells after adoptive transfer. A, at day 4 and day 11 after the last T-cell injection, single-cell suspensions were obtained from spleens of two 3AB11-treated mice and one untreated mouse at both time points and subjected to FACS analysis. Human CD45 and CD3 are depicted for the cell population gated on eGFP (tumor) negative and murine CD45 negative. B, CTL_{TT}-TCR–treated mice were injected with TT-loaded or unloaded EBV-LCL cells (15×10^6 i.v. and 2×10^6 s.c. where indicated). T cells were *in vivo* labeled using α-huCD4-ALEXA-700 antibody and visualized on the dorsal side of anesthetized mice using fluorescence imaging. C, in a second experiment at day 43 after T-cell injection, TT-loaded EBV-LCL cells (15×10^6) were i.v. injected, and TT-loaded or unloaded EBV-LCL cells and UM9 cells (2×10^6) were s.c. injected at indicated locations. T cells were visualized using fluorescence imaging. D, treatment of extramedullary relapses by additional injections of 3AB11. Established UM9-luc-eGFP tumors in bone marrow of $Rag2^{-/-}\gamma c^{-/-}$ mice were treated with 3AB11 ($n = 4$; ○). Twenty-two days after primary treatment, the mice were again i.v. injected with 3AB11. Arrows, T-cell injections. Mean and SE of tumor photon emission are shown. E, resistance of extramedullary tumors to intratumoral treatment with 3AB11. $Rag2^{-/-}\gamma c^{-/-}$ mice were s.c. inoculated with 9×10^6 UM9-luc-eGFP cells. Three weeks after inoculation, the tumors were treated by intratumoral injections of 3AB11 (12×10^6 cells per tumor; $n = 5$; ○; arrow). TT-specific T cells CTL_{TT} ($n = 5$; ●) or PBS ($n = 6$; ■) were injected in different tumors as control. Tumor progression was monitored by BLI and mean tumor photon emission is shown.

support that the adoptive T-cell therapy was compromised within the extramedullary microenvironment by local regulatory factors inhibiting the efficacy of T cells.

Discussion

We here show for the first time the *in vivo* cytotoxic antitumor effect of sole CD4$^+$ human T cells recognizing a single multiple myeloma–associated mHag. We also show that direct antitumor effects can be achieved by adoptive immunotherapy using recall antigen (TT)-specific CD4$^+$ cytotoxic T cells that are genetically engineered to express the TCR recognizing the same single mHag. However, our specific CD4$^+$ T-cell therapy was not protective against relapse of multiple myeloma at extramedullary sites, which is also a known complication in patients treated with DLI

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(23–26). Our results are relevant to improving cellular antitumor immunotherapy in three ways:

First of all, our results show an important and additional role of CD4+ T cells in anticancer immunity. Currently, the generally entertained idea is that the CD4+ T cells contribute to anticancer immunity mainly via activation of and support to other immune cells (7, 10, 11, 27–30). This current dogma remains despite a substantial number of studies showing that Th1-like CD4+ T cells, especially of human origin, often display cytotoxic activity against relevant tumor cells (12–15). This resistance to recognize the importance of CD4+ T cell–mediated tumor cytotoxicity is likely due to the fact that most of the previous cytotoxicity studies were done *in vitro* but not *in vivo*. Furthermore, in other *in vivo* studies involving CD4+ T cells, other effector cells were present, frequently preventing determination of the sole contribution of CD4+ T cells in clinical outcome. Fortunately, recent elegant murine studies have shown that naive tumor/self-specific CD4+ T cells can naturally differentiate into Th1 cytotoxic T cells *in vivo* and can cause regression of established melanoma independent of CD8+ T, B, and NK cells (16, 31). In line with these observations for CD4+ murine T cells, we here provide a strong indication for the establishment of an anti–human tumor effect due to direct cytotoxic effector function of CD4+ human T cells in a model lacking T, B, and NK cells and depleted of macrophages prior to T-cell infusions. Thus, in the light of our results, we think that CD4+ T cells deserve to be included in clinical trials alone or in combination with other effector cells.

Second, extending the results of several previous reports in murine experimental models (reviewed by Bendle et al.; ref. 32), our results show that the TCR-transfer approach is also a highly valuable method to generate therapeutic CD4+ human T cells, as they, like their parental cells, exerted effective antitumor effects in our model. A potential advantage of inserting therapeutic TCRs into TT-specific T cells may be the possibility to boost these therapeutic T cells via their intrinsic TCRs. Indeed, we showed the reactivation of long-term persisting dual antigen–specific T cells by injections of TT-loaded antigen-presenting cells; nonetheless, our model did not allow us to test the potential advantage of this strategy because our original sole CD4+ T-cell injections were (*a*) highly effective against tumors residing in bone marrow but (*b*) could not control extramedullary relapses.

Finally, an important observation in our study is the discrepancy between the responses of bone marrow versus extramedullary tumors after CD4+ cellular immunotherapy. We have been able to provide an optimal visualization of this phenomenon, because unlike several other tumor models, our bioluminescence model allows quantitative detection of individual tumor loci at various tissues. In our model, mice relapsed approximately 40 days after tumor injection, comparable with recurrences seen at the original tumor site in several other models after an initial effective T cell–based therapy (33–37). Although we have not been able to elucidate the exact mechanism of these extramedullary relapses, we ruled out several known tumor escape mechanisms. Our results clearly show that the extramedullary escape is not due to antigen or MHC downregulation or development of an intrinsic resistance toward T cell–mediated cytotoxicity because extramedullary tumors showed no *ex vivo* resistance toward lysis by mHag-specific T cells (see Fig. 3D). In further experimentation we also ruled out mechanisms of impaired T-cell survival *in vivo* or defective T-cell homing to extramedullary sites (see Fig. 4). Additional i.v. therapy or even intratumoral therapy of extramedullary tumors were also ineffective. It has been shown by others that although T cells could traffic towards extramedullary sites, the tumors present at those sites may still escape if the T cells fail to infiltrate them (38). Another possibility for extramedullary tumor escape is the inactivation of T cells due to local secretion of inhibitory factors. Such an inhibitory factor could be the vascular endothelial growth factor, because its expression in ovarian cancer shows an inverse correlation with the number of T cells infiltrating into the tumor microenvironment and it inhibits T-cell effector functions (39, 40). In fact, many other soluble factors such as transforming growth factor β, prostaglandin E2 and interleukin-10, as well as inhibitory ligands such as PD-L1/2, CTLA-4 ligands, FASL, and tumor necrosis factor–related apoptosis-inducing ligand have been shown to contribute to T-cell suppression within the tumor microenvironment (41–43). We are currently investigating the potential role of such mechanisms in our model by defining surface phenotype and function of transferred T cells at extramedullary sites. Furthermore, it may be necessary to investigate whether extramedullary relapses can be prevented by combination of CD4+ human T cells with other effector cells of adaptive and innate immunity, such as CD8+ T cells or NK cells.

In conclusion, our study indicates that native as well as TCR-transduced CD4+ human T cells can significantly contribute to antitumor immunity via their cytotoxic capacity, especially against tumors residing in the bone marrow. These results encourage the evaluation of their immunotherapeutic potency in clinical phase I/II trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Louis van Bloois of the Department of Pharmaceutics, University of Utrecht, for generating CL2MDP liposomes, and Justin Kline and Fred Locke for stimulating discussions and critical reading of the manuscript.

Grant Support

University Medical Center Utrecht, the Netherlands.

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Received 05/18/2010; revised 09/18/2010; accepted 09/28/2010; published OnlineFirst 11/09/2010.

Published OnlineFirst November 14, 2010; DOI: 10.1158/1078-0432.CCR-10-1340

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Published OnlineFirst November 14, 2010; DOI: 10.1158/1078-0432.CCR-10-1340

Clinical Cancer Research

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Clin Cancer Res 2010;16:5481-5488. Published OnlineFirst November 14, 2010.