Definition of an Enhanced Immune Cell Therapy in Mice That Can Target Stem-Like Lymphoma Cells

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Abstract

Current treatments of high-grade lymphoma often have curative potential, but unfortunately many patients relapse and develop therapeutic resistance. Thus, there remains a need for novel therapeutics that can target the residual cancer cells whose phenotypes are distinct from the bulk tumor and that are capable of reforming tumors from very few cells. Oncolytic viruses offer an approach to destroy tumors by multiple mechanisms, but they cannot effectively reach residual disease or micrometastases, especially within the lymphatic system. To address these limitations, we have generated immune cells infected with oncolytic viruses as a therapeutic strategy that can combine effective cellular delivery with synergistic tumor killing. In this study, we tested this approach against minimal disease states of lymphomas characterized by the persistence of cancer cells that display stem cell–like properties and resistance to conventional therapies. We found that the immune cells were capable of trafficking to and targeting residual cancer cells. The combination biotherapy used prevented relapse by creating a long-term, disease-free state, with acquired immunity to the tumor functioning as an essential mediator of this effect. Immune components necessary for this acquired immunity were identified. We further demonstrated that the dual biotherapy could be applied before or after conventional therapy. Our approach offers a potentially powerful new way to clear residual cancer cells, showing how restoring immune surveillance is critical for maintenance of a disease-free state. Cancer Res; 70(23): 1–9. ©2010 AACR.

Introduction

We recently described a novel combination approach for the treatment of cancer (1), utilizing a tumor-targeting immune cell line [cytokine-induced killer (CIK) cells; refs. 2, 3] as a carrier vehicle to systemically deliver an oncolytic vaccinia virus (vvDD; refs. 4–6) to tumors. It was found that these 2 therapies could synergize in their tumor targeting and killing in large primary tumors, for which immune cell therapies are typically less effective (7). Here, we sought to examine the ability of this dual biotherapy to treat minimal lymphoma disease states, characterized by the presence of residual cancer cells with stem cell–like properties and increased resistance to conventional therapies. Because the last remaining cancer cells following treatment, or those that initiate metastasis, are likely to represent a heterogeneous population distinct from the cell types that constitute the majority of the tumor, therapies that target and destroy cancer cells by multiple distinct mechanisms such as the CIK-vvDD dual biotherapy will be needed to eradicate them. We therefore sought to determine for the first time, whether CIK-vvDD dual biotherapy could specifically target and destroy residual disease in addition to the bulk tumor. This property would potentially make this approach a powerful, new tool in the prevention of relapse.

We incorporated a model of residual disease, using lymphoma cells isolated from spontaneous tumors formed in a transgenic mouse model utilizing the tet-OFF system, to conditionally regulate MYC oncogene overexpression in the lymphoid compartment (8–13). These tumors regress upon MYC oncogene inactivation. However, sustained MYC inactivation results in a small population of cells capable of persisting and eventually relapsing subsequent to chromosomal rearrangement (14). These cells, therefore, have some stem cell–like or tumor-initiating properties. This model allowed the reliable and reproducible creation of a minimal disease state after tumor regression that resulted in relapse if left untreated. These conditions could not be recreated with standard xenografts models.

Here, we demonstrate that CIK cells can target these cells, even after MYC inactivation in vitro and in vivo, and that
CIK-vvDD dual biotherapy can clear the residual cells and induce an acquired immune response that prevents tumor relapse. This approach may, therefore, be applied to greatly enhance the effectiveness of conventional cancer therapies in a variety of settings and represents a novel means to directly target cancer-initiating cells.

Materials and Methods

Cells and viruses

Lymphoma cell lines 6780, 6814, and 8946 have been described previously (14) and were obtained from a spontaneous tumor transgenic mouse model incorporating the tetracycline transactivating protein (tTA) driven by the immunoglobulin heavy chain enhancer element and the SRalpha promoter (SR-tTA) and the tetracycline-responsive minimal promoter (tet-o) driving MYC proto-oncogene elements, such that oncogenic MYC is overexpressed in the absence, but not in the presence, of doxycycline exclusively in hematopoietic cell lineages (9). Cells isolated from spontaneous tumors (primarily B-cell lymphomas) formed in these mice can be transferred into naive mice to form tumors that will regress when doxycycline is present (14).

The cells were labeled with firefly luciferase by retroviral infection and luciferase expression verified. Cell morphology, growth rates, and response to doxycycline exposure were compared with the unlabeled parental strains.

The preparation of CIK cells [an ex vivo expanded NK-T cell population (3, 15)] has been previously described (2). Briefly, mouse splenocytes or human peripheral blood lymphocytes were treated with IFN-γ for 24 hours before exposure to anti-CD3 antibody for 24 hours and expansion in the presence of IL-2 for a period of 14 to 21 days. CIK cells expressing luciferase were produced from an FVB transgenic strain expressing luciferase from the β-actin promoter [as previously described (16)].

The viral strains used in this work were produced from Western Reserve strain of vaccinia, with mutations in the viral thymidine kinase and viral growth factor genes (6). Versions of the resultant double deleted virus (vvDD) expressing either luciferase or green fluorescent protein from a synthetic early/late viral promoter have been described previously (1). To produce the CIK-vaccinia dual biotherapy, CIK cells and vvDD were mixed at a multiplicity of infection of 1.0 for 5 hours prior to use.

Cell viability assays

In some experiments, cell lines were treated with increasing doses of irradiation or chemotherapy (paclitaxel; Sigma); 7 days after irradiation or 3 days after addition of chemotherapy, cell viability was determined by MTS assay (Promega).

Animal models

Tumors were formed by implantation of lymphoma cells either subcutaneously (1 × 10^6 cells) or into the peritoneal cavity (5 × 10^6 cells) of FVB or NOD SCID mice (Jackson Laboratories). Tumor growth was determined by caliper measurement or in vivo bioluminescence imaging (BLI, see later), with treatment initiated either once primary tumors had established (reached 100 mm^3, or had displayed increasing BLI signal for 2 consecutive images) or at time points as described in the legends to figures. Tetracycline-mediated regression of tumors was achieved by the addition of doxycycline (50 ng/mL) to the drinking water (water changed every 24 hours). In some experiments, BALB/c mice were injected subcutaneously with 1 × 10^7 A20 lymphoma cells (ATCC).

Animals were treated with 1 × 10^7 plaque forming units (pfu) of vvDD; 1 × 10^6 CIK cells, or 1 × 10^7 pfu of vvDD premixed with 1 × 10^7 CIK cells via tail vein injection. In some experiments, vvDD and CIK cells were delivered together without the preinfection step. Cyclophosphamide was delivered through intraperitoneal injection (100 mg/kg).

BLI was carried out using an IVIS200 system (Caliper LifeSciences) following intraperitoneal injection of 200 μL of 30- mg/mL luciferin substrate and anesthesia with 3% isoflurane. Images were analyzed using LivingImage software (Caliper LifeSciences).

Immune assays

In the cytotoxic T-lymphocyte (CTL) kill assay, CIK effector cells were mixed with lymphoma target cells expressing luciferase in effector to target ratios of 5:1 to 50:1 in triplicate and incubated for 12 hours. Target cell survival was measured by BLI and compared with target cells alone (100% viable) or effector cells alone (0% viable).

The degranulation assay was performed as previously described (17). Briefly, lymphocytes were mixed with antigens in the presence of GolgiStop (BD Biosciences) and anti-CD107a-FITC antibody or anti-CD154-PE (BD Pharmingen). After 6 hours, cells were washed and counterstained with CD8-PE and CD4-FITC, respectively. Percentages of CD4 or CD8 cells undergoing degranulation (i.e., CD154 or CD107a cell surface stains, respectively) in response to different stimuli were quantified by flow cytometry (FACScalibur; BD Pharmingen).

Cytokine levels were assessed via intracellular staining. Lymphocytes were mixed with target antigens in the presence of GolgiStop. Cells were fixed, permeabilized (BD cytofix/cytoperm), and stained with conjugated antibodies to IL-4, IL-10 (both from Biolegend), IFN-γ (ebioscience), or IL-2 (Caltag), as well as surface stains to CD4 and CD8. Percentages of CD4 or CD8 cells staining positive for different cytokines were determined by flow cytometry.

Antivaccinia-neutralizing antibody titers were determined in plasma samples obtained from treated mice. Serial dilutions were mixed with 1,000 pfu of vvDD-luciferase virus for 2 hours, before being transferred to A2780 cells (ATCC) in 96-well plates. Luciferase signals from vaccinia-infected cells were determined after 24 hours. Neutralizing antibody levels were determined as the dilution that resulted in neutralization of 50% of the luciferase-expressing virus.

ELISAs were run according to the manufacturer’s instructions (IP-10 and I-TAC ELISA; R&D Systems).

qRT-PCR was run on cDNA samples obtained from cells; collection and processing of cDNA were according to...
manufacturer's instructions, and qRT-PCR was run in 96-well array formats using commercial kits (Th1-Th2-Th3 kits; SuperArray, part of SA Biosciences).

The cells were also stained for several additional surface markers before analysis by flow cytometry. These included MULT-1 (R&D Systems), Rae-1 (BD Pharmingen), and Sca-1 (stem cell antigen-1; BD Pharmingen).

**Statistical analysis**

Unpaired and paired Student's t tests were run to determine statistical significance (defined as P < 0.05).

**Results**

**In vitro and in vivo targeting of lymphoma cell lines by CIK cells is possible even after MYC inactivation**

MYC inactivation of lymphoma cells cultured from spontaneous tumors formed in the EμSR-tTA tet-o-Myc transgenic mouse in vitro leads to cell-cycle arrest, reversion to a "normal" lymphocytic morphologic phenotype, and eventually senescence and apoptosis (14). We found MYC inactivation (50 mg/mL of doxycycline) and differentiation back into a premalignant phenotype did not result in evasion from CIK-mediated killing for any of the cell lines tested (6780, 8946, and 6814) (Fig. 1A). CIK cells efficiently killed the lymphoma cells after loss of the neoplastic phenotype but did not kill normal lymphocytes (data not shown). Because CIK cells recognize and destroy their tumor targets through recognition of NKG2D ligands (Rae-1, H-60, or MULT-1 in the mouse), we looked to determine the levels of cell surface expression of these ligands with and without MYC expression. Although different lymphomas from the same transgenic strain displayed different NKG2D ligand expression patterns and the loss of MYC expression generally resulted in reduction in cell surface levels of these ligands, significant levels of MULT-1 were typically maintained (Fig. 1B).

MYC repression in vivo resulted in lymphoma regression, and implanted tumors become undetectable after 14 to 21 days, but relapses frequently occur after sustained repression (of up to 30 weeks), implying that residual tumor cells are retained for...
extended periods. We used BLI to determine that CIK cells were equally effective at trafficking to the site of a regressed tumor as they were at trafficking to primary tumors and could target the minimal residual disease states whether MYC expression was reactivated or was continually repressed (Fig. 1C).

CIK-vvDD dual biotherapy prevents relapse from residual lymphoma cells persisting after MYC inactivation

Mice with intraperitoneal 6780 lymphoma cell line underwent MYC oncogene inactivation, leading to tumor regression (Fig. 2A) until it was no longer detectable by imaging (indicating <1,000 tumor cells remained; Supplementary Fig. S1). MYC inactivation was continued for a further 7 days and then animals were intravenously administered with i) phosphate buffered saline (PBS), ii) vvDD, iii) CIK cells, iv) CIK cells and vvDD, or iv) CIK-vvDD dual biotherapy (vvDD preinfected into CIK cells). Immediately after treatment, MYC expression was reintroduced. Animals treated with PBS reproducibly relapsed after 7 to 10 days (Fig. 2A and B). This system therefore represents an experimental model of therapeutic clearance of detectable tumor, with a few remaining tumor cells leading to relapse. Because of the small number of lymphoma cells mediating this relapse, they must have tumor-initiating properties. Animals in this minimal disease state treated with CIK cells alone demonstrated a relapse rate of 85% (6 of 7) with a general delay to relapse (from an average of 8 to 27 days; Fig. 2B). Interestingly, this prolongation of disease-free state corresponded to the approximate period of time (about 21 days) we expect the CIK cells to remain resident within the host (18). It was found that repeat treatment with a second dose of CIK cells 14 days after initial treatment could further delay the average time to relapse to 48 days (Fig. 2C), but overall survival did not improve. Animals treated with a single intravenous injection of oncolytic virus demonstrated a similar overall rate of relapse (6 of 7) as CIK treatment. However, in this group, there was typically no delay to relapse, indicating that the virus, which cannot actively target tumor cells, could not reach the residual disease in
the majority of the animals. Animals treated with both CIK and vvDD displayed similar relapse kinetics as CIK cells alone. Finally, animals treated with the dual biotherapy (CIK-vvDD) displayed a significantly lower rate of relapse (3 of 7, \( P = 0.001 \)). It therefore appears that the ability of the CIK cells to home to the residual tumor cells coupled with the additional killing mechanisms provided by delivery of oncolytic virus resulted in greatly improved clearance of the minimal residual.

**Lymphomas capable of relapsing from minimal disease states also display increased resistance to radio- and chemotherapy**

In a second lymphoma cell line (6814; isolated from a second mouse from the same transgenic strain), it was found that subsequent to regression, only 5 of 7 of the PBS-treated mice actually relapsed once MYC expression was resumed (Fig. 3A). It therefore appears these cells possess reduced stem cell–like qualities. Although no defined “cancer stem cell” or cancer-initiating cell markers exist for murine lymphomas, it was found that Sca-1 was upregulated on the surface of the 6780 lymphoma cell line that reliably relapsed relative to the 6814 cell line (Fig. 3B). In this second tumor model, only 1 of 7 CIK-treated mice, 2 of 7 vaccinia-treated, and 0 of 7 dual biotherapy–treated animals relapsed (Fig. 3A). Interestingly, the Sca-1\(^{hi}\) 6780 cell line, which displays the greatest rates of relapse, was also found to be significantly more resistant to both radiotherapy and chemotherapy (paclitaxel treatment) than the 6814 cell line and so possesses other properties often associated with tumor-initiating cells (Supplementary Fig. S2).

**Prevention of relapse and the creation of a long-term disease-free state by CIK-vvDD therapy require an adaptive immune response**

When the experiment in Fig. 2A was repeated using the 6780 cell line implanted into NOD SCID mice, PBS treatment resulted in the same timing and rate of relapse as those in the immunocompetent animals (Fig. 3C). However, when residual disease in immunodeficient mice was treated with CIK-vvDD dual biotherapy, treated animals displayed a delay to relapse but did all relapse, closely mirroring the response of the CIK-treated group in immunocompetent animals. This implies that the ability to create a long-term, disease-free state is dependent on an intact host immune response and surprisingly is not simply due to the additional tumor-killing ability provided by vvDD. We therefore looked to determine whether mice displaying complete responses following CIK-vvDD dual biotherapy could reject rechallenge. Mice bearing the 6780 or 6814 tumor cell line (from Figs. 2 and 3A) that had demonstrated long-term (>90 days), disease-free states after treatment were rechallenged with the same cell line. It was found that the tumors initially treated with CIK-vvDD dual biotherapy could reject rechallenge (Fig. 3D and Supplementary Fig. S3). It was also seen that the (Sca-1\(^{lo}\) 6814 tumor cell line tumors that did not relapse in the PBS control group were susceptible to rechallenge (Supplementary Fig. S3), confirming that a lack of regenerative qualities in this cell line is the cause of the lower rates of relapse rather than any increased immunogenicity of these tumor cells. Single therapies in this tumor model that led to complete responses were also typically not protective.
To determine the basis of the immune involvement in rejection of rechallenge, animals were left for a further 90 days before the remaining animals (n = 3 with the 6780 tumor cell line) were sacrificed. Splenocytes recovered from these mice were exposed to the same lymphoma cells *ex vivo* and numbers of CD4<sup>+</sup> and CD8<sup>+</sup> cells recognizing antigens on these cells were determined by degranulation assay. Increased levels of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> splenocytes recognizing the tumor antigens (relative to splenocytes from naive controls) indicated the presence of a long-term memory T-cell response (Fig. 4A).

In a correlative experiment, animals with a large primary 6780 tumor cell line implanted into the peritoneal cavity were treated with single intravenous injections of PBS, CIK cells, vvDD, or CIK-vvDD dual biotherapy at noncurative doses. Although the dual biotherapy–treated group displayed the greatest response to treatment (Fig. 4B), ultimately all animals displayed tumor regrowth. As before, tumors regressed to undetectable levels by BLI before the MYC oncogene was reactivated (to model a subsequent curative therapy). In a second experiment, intracellular cytokine staining was used to determine the levels of Th1-associated cytokines (IL-12 and IFN-γ) and Th2-associated cytokines (IL-4 and IL-10) produced by CIK cells. It was found that CIK cells alone produced high levels of IFN-γ, low levels of IL-12 and IL-10, and no IL-4 (Fig. 5B), whereas CIK cells preinfected with vvDD produced increased levels of IFN-γ and IL-12, skewing the response toward the Th1 arm and explaining why preinfected CIK cells were better able to induce a cellular antitumor immune response than were CIK cells and vvDD alone.

Preinflection of CIK cells with vvDD, leading to a Th1 skewing of the immune response, is critical for optimizing the induction of antitumor immunity

The presence of CIK cells colocalized with vaccinia infection therefore seems to direct the immune response. This was further tested; first, the immune response targeting the virus itself was examined after the treatment of subcutaneous 6780 tumor cell line with vvDD or vvDD-CIK. It was seen that levels of CD4<sup>+</sup> cells capable of degranulation in response to virus *ex vivo* (CD154 positive) were increased when vvDD-CIK treatment was used relative to vvDD alone and that the levels of neutralizing antibody produced were decreased under the same conditions (Fig. 5A). The immune response induced against the virus therefore seemed skewed toward a Th1 response when CIK cells were present. Effective induction of antitumor immunity may be effective in an adjuvant setting before debulking surgery or chemotherapy.

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virus delivered together without the preinfection step. A qRT-PCR array was also performed with a panel of Th1/Th2 cytokines. This confirmed that CIK cells produced more IL-12 and IFN-\(\gamma\) than IL-4 and IL-10 (data not shown). Overall, very few genes were upregulated as a result of viral infection of CIK cells and the only genes significantly upregulated included IL-2, CD28, and IL-7, indicating again that preinfected CIK cells may act as more potent instigators of a cellular adaptive immune response. Finally, we looked at the production of chemokines known to attract Tc1 cells through binding to CXCR3 (CXCL10 (IP-10) and CXCL11 (I-TAC)) (Fig. 5C). It was found that CIK cell production of CXCL11 was increased when the cells were infected with vvDD. CXCL10 was only weakly produced by infected or uninfected CIK cells. However, when the CIK cells (with or without viral infection) came into contact with lymphoma cells, significant amounts of this chemokine were produced. It would therefore appear that the combination of cytokines and chemokines produced when infected CIK cells are located within the tumor would induce a cellular (Th1) host immune response.

CIK-vvDD dual biotherapy also functions to clear minimal disease states produced following chemotherapy treatment

We looked to extend these observations beyond the tet-Off–regulated MYC system and model a more clinically accurate system. BALB/c mice were therefore implanted with A20 lymphoma cells and treated with intravenous injection of vvDD; CIK or vvDD-CIK either 2 days before or 14 days after chemotherapy (intraperitoneally injected cyclophosphamide 100 mg/kg, as a common lymphoma treatment; Fig. 6). When the chemotherapy was applied first (Fig. 6A), the biological therapies were applied at a point when tumors had regressed considerably from the time of initial treatment but regrew without any additional therapy. As before, the CIK and vvDD therapies used alone slowed or delayed, but could not prevent, tumor reformation. vvDD-CIK dual biotherapy was again the most effective, and 8 of 8 mice remained disease free despite the known immunosuppressive nature of cyclophosphamide.

When the biological agents were added first (Fig. 6B), modeling an adjuvant setting, single therapies (CIK or vvDD alone) resulted in only very minor additional benefit over the che-
motherapy alone. The dual biotherapy again led to the greatest overall therapeutic benefit (with 3 of 8 complete responses).

**Discussion**

Therapies designed specifically to target the minimal disease remaining after a successful therapy or debulking surgery might provide a dramatic improvement in life expectancy for patients with cancers who are prone to relapse. However, such therapies must be able to both reach the last cancer cells and destroy these cells, which are frequently heterogeneous and phenotypically distinct from the bulk tumor. Any therapies specifically designed to target these cells must therefore have the ability to destroy tumor cells through multiple mechanisms.

Here, we extend our previous work targeting bulk tumors with a novel dual biotherapy (vvDD-CIK) to demonstrate that this therapeutic approach displays a profound ability to specifically clear the last remaining tumor cells and thus prevent relapse. We also demonstrate for the first time that the critical factor for this process is the immunogenicity of the virus, aided by the release of cytokines and chemokines from the CIK cells that together can lead to potent CTL responses as an additional antitumor mechanism.

Because minimal residual disease is traditionally difficult to reliably and reproducibly recreate in preclinical models, we developed a novel system to test this therapy. In this model, regulated inactivation of oncogenic MYC leads to reversion of tumor cells to an undifferentiated phenotype *in vitro* and tumor regression *in vivo*. Subsequent reactivation of MYC expression leads to tumor relapse despite the residual number of tumor cells being very low (<1,000). It was also found that an ability to reform tumors from a minimal disease state was not a universal property of spontaneous tumors formed in this transgenic mouse model but did correlate with surface expression levels of the Sca-1 and increased resistance to both chemotherapy and radiotherapy.

CIK-vvDD biotherapy prevented relapse in more than half of the mice with a minimal disease state. Interestingly, CIK cells alone could delay relapse, but once they were no longer present, the cancer returned. It would seem likely that the increased tumor-killing potential provided by vvDD released from CIK cells would be responsible for this ability to clear the last remaining cancer cells; however, the fact that no such enhanced effect was seen in immunodeficient mice surprisingly implies that the additional benefits are in fact mediated by the host immune response. This represents for the first time a situation under which the directly oncolytic potential of the virus is not necessary, with its immunogenic potential being of paramount importance instead. This is not entirely unexpected, however, as others and we have reported that oncolytic viruses are capable of transitioning to a predominantly immune-mediated mechanism of tumor killing under some conditions (19). As CIK-mediated delivery of vvDD may not allow direct targeting of every remaining cancer cell by the therapeutic, the additional immune response raised against tumor antigens would be necessary for complete tumor clearance and long-term immune surveillance to prevent relapse.

It was further demonstrated that previously undescribed and unique characteristics of CIK cells infected with vvDD lead to the greatest induction of CTL and likely also enhance the trafficking of these T cells to the residual tumor cells. In particular, a Th1 skewing of the immune response and increased levels of chemokines known to attract activated T cells were produced by, or in response to, CIK cells infected with vvDD.

Finally, it was shown that it was also possible to induce the immune response through treatment of bulk tumors but that the benefits of this immune response were not seen until after the large (and presumably immunosuppressive) tumors had been removed. This implies that this approach can be applied in either an adjuvant or a neoadjuvant setting. This was confirmed in a second mouse model of residual disease, involving chemotherapy treatment to debulk lymphoma.

CIK-vvDD treatment of minimal residual disease, or even prior to other debulking therapeutic strategies, therefore represents a novel approach for the treatment of cancer patients and a novel application for CIK-vvDD therapy, as well as providing a much needed means to clear the last remaining cancer cells that possess tumor-initiating
properties and an increased resistance to chemotherapy and radiotherapy.

Disclosure of Potential Conflicts of Interest

S.H. Thorne holds stock in Jennerex Biotherapeutics. The other authors disclosed no potential conflicts of interest.

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