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Engineered drug-resistant immunocompetent cells enhance tumor cell killing during a chemotherapy challenge

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ABSTRACT

Establishment of immunocompetent cell mediated anti-tumor immunity is often mitigated by the myelosuppressive effects during administration of chemotherapy. We hypothesized that protecting these immune cells from drug induced toxicities may allow for the combined administration of immunotherapy and chemotherapy. Using a SIV-based lentiviral gene transfer system we delivered the drug-resistant variant P140KMGMT into the immunocompetent cell lines NK-92 and TALL-104, and the myelogenous leukemia cell line, K562, which is a target for both NK-92 and TALL-104 cells. Genetically engineered immunocompetent cells developed significant resistance to temozolomide compared to non-modified cells, and genetic modification of these cells did not affect their ability to kill K562 cells. We then evaluated the effectiveness of drug-resistant immunocompetent cell mediated killing of tumor cells in the presence and absence of chemotherapy. During a chemotherapy challenge the cytotoxic activity of non-modified immunocompetent cells was dramatically impaired. However, when combined with chemotherapy, genetically-modified immune cells retained their cytotoxic activities and efficiently killed non-modified target cells. These results show that engineering immunocompetent cells to withstand chemotherapy challenges can enhance tumor cell killing when chemotherapy is applied in conjunction with cell-based immunotherapy.

Introduction

Although outstanding progress has been made in the fields of cancer detection and tumor cell biology, the treatment of late-stage and metastatic cancer remains a major challenge. Cytotoxic chemotherapy agents remain among the most used and successfully employed anti-cancer treatments. However, they are not uniformly effective, and the introduction of these agents with novel therapies, such as immunotherapies, is problematic. For example, chemotherapy agents can be detrimental to the establishment of robust anti-tumor immunocompetent cells due to their non-specific toxicity profiles. Current clinical trials employing small molecules that target proliferative pathways may also hamper the establishment of anti-tumor immunity. However, if chemotherapy regimens that are transiently effective can be combined with novel immunocompetent cell therapies then significant improvement in anti-neoplastic therapy can be achieved. Here we focused on the development of methods whereby immunocompetent cells are protected from the toxic effects of chemotherapy, thereby allowing co-administration of chemotherapy and cell-based immunotherapy, a treatment we have termed drug-resistant immunotherapy.

Several drug-resistant genes have been identified that can potentially be used to confer drug resistance to targeted cells, and advances in gene therapy techniques have made it possible to test the feasibility of using these genes in drug resistance gene therapy studies [1–7]. In addition, a shRNA strategy was used to decrease the levels of hypoxanthine–guanine phosphoribosyltransferase, which conferred resistance to 6-thioquanine [8]. Among the drug-resistant genes studied, MGMT is among the most promising. This gene encodes for human alkyl guanine transferase (hAGT), a DNA repair protein that confers resistance to the cytotoxic effects of alkylating agents, such as nitrosoureas and temozolomide (TMZ). 6-Benzylguanine (6-BG) is an inhibitor of AGT that potentiates nitrosourea toxicity and is co-administered with TMZ to potentiate the cytotoxic effects of this agent. Several mutant forms of MGMT that encode variants of AGT are highly resistant to inactivation by 6-BG, but retain their ability to repair DNA damage [9]. P140KMGMT-based drug-resistant gene therapy has been shown to confer chemoprotection to mouse, canine, rhesus macaques, and human cells, specifically hematopoietic cells [7,10–14]. We hypothesized that the protection of immune-effector cells by the introduction of P104KMGMT can allow for the co-administration of chemotherapy and immunotherapy to mediate an improved anti-cancer therapeutic response. The aim of this study was to evaluate the feasibility of using a drug-resistant immunotherapy strategy employing two genetically altered immu-

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nocompetent cell lines, NK-92 and TALL-104, by lentiviral transfer of the cDNA sequence encoding for P140KMGMT.

The highly potent cytotoxic human NK cell line, NK-92 is an interleukin-2 (IL-2)-dependent human natural killer cell line with functional and phenotypic characteristics of activated NK cells [15]. NK-92 cells are effectors of the innate immune system, which play an important role in host responses against viruses and tumor cells. Due to the high cytotoxicity against a broad spectrum of primary and established tumor cells at low effector:target ratios and against primary leukemia in SCID mice [15–17], these cells are a reasonable candidate to use as a drug-resistant immune-effector cell [16,17]. TALL-104 cells are an interleukin-2-dependent leukemic T cell line that has surface markers typical of both cytotoxic T lymphocytes and natural killer cells, and lyse tumor cells in a non-HLA-restricted fashion [18]. Adoptive immunotherapy with TALL-104 cells has induced long-term complete or partial remissions in tumor bearing animals [18,19]. Because these cells can be expanded in culture indefinitely, similar to NK-92 cells, we used TALL-104 cells as immunocompetent cells in these proof-of-concept experiments.

In the present study, the immunocompetent cell lines NK-92 and TALL-104 were genetically engineered to express P140KMGMT, and their chemoprotection against 6-BG/TMZ was assessed. The cytotoxicities of the drug-resistant cells against the tumor cell line, K562, were then accessed in the presence of 6-BG/TMZ. Our findings indicate that, compared to wild-type cells, both gene-modified immune cell lines mediate enhanced cytotoxicities, albeit in varied degrees, in the presence of drug, which supports the concept that drug-resistant immunotherapy is a potential anti-cancer therapy.

Materials and methods

Cell cultures. Human cell lines, NK-92 and TALL-104, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained as described before [15,18].

Generation and titering of recombinant retrovirus. The cDNAs encoding for human P140KMGMT or eGFP were PCR amplified using appropriate primers and inserted into an SIV expression vector, and a four plasmid system (kindly provided by Dr. Arthur Nienhuis) was used to generate recombinant SIV-lentivirus. Transient transfection was carried out in 293T producer cells using methods as previously described [20,21]. The titers of virus encoding eGFP and P140KMGMT were determined by flow cytometry and real-time polymerase chain reaction (PCR), respectively [20,21].

Lentiviral transduction. Transductions of SIV-based lentiviral particles were performed by incubating cells with virus in cell growth medium supplemented with polybrene (6 μg/ml; Specialty Media, Phillipsburg, NJ). Twenty-four hours post transduction, virus-containing medium was replaced with fresh medium and transduced cells were cultured until reaching approximately 70–90% confluency (i.e. 48 h or longer), at which point cells were used for downstream applications.

Survival curve analysis. Non-modified and P104KMGMT-modified cells were exposed for 2 h to 6-BG (25 μM), then to increasing concentrations of TMZ for 48 h. At this time cell viability was assessed by trypan blue exclusion method. All drugs were freshly prepared on the day of use. Each experiment was performed a minimum of three times.

Cytotoxicity assay. Cells were grown in the presence of 100 U/mL recombinant human IL-2, exposed to 6-BG (25 μM) for 2 h, followed by the addition of TMZ (200 μM) and overnight incubation. To determine the effector cell (*E*) concentration that resulted in maximal killing of the target cells (*T*), 4000T cells were placed in 96-well plates and mixed with effector (*E*) cells (NK-92 or TALL-104 cells) at *E*:*T* ratios of 2.5:1, 5:1, and 10:1 (in triplicate) followed by a 4 h incubation. The amount of LDH released to the supernatant as a result of cytolysis of target cells was measured using a lactate dehydrogenase (LDH) release assay according to the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Cytotoxicities were expressed as % cytotoxic activity of the effector cells and were calculated as:

$$\%\ \text{Cytotoxicity} = \frac{(\text{Experimental release} - \text{Spontaneous release}_{\text{effector}}) - \text{Spontaneous release}_{\text{target}}}{\text{Maximum release}_{\text{target}} - \text{Spontaneous release}_{\text{target}}} \times 100$$

Results

Lentiviral transduction efficiencies of NK-92, TALL-104 and K562 cells

The transduction efficiency of NK-92, TALL-104 and K562 cells were initially evaluated using a self-inactivating, VSV-G pseudotyped, recombinant SIV-lentivirus, encoding eGFP (Fig. 1A). The expression of eGFP was driven by the murine stem cell virus LTR promoter. To measure the transduction efficiencies, all cell lines were transduced with a multiplicity of infection (MOI) of 40, and GFP fluorescence was analyzed by flow cytometry at 72 h after transduction. Transduction of each cell line resulted in robust expression of eGFP (as visualized by fluorescence microscopy) which was quantitated as 90%, 41% and 99% in NK-92, TALL-104 and K562 cell lines, respectively (Fig. 1B–D). Thus, high transduction efficiencies were achieved for both NK-92 and K562 cell lines, and TALL-104 cell lines exhibited moderate transduction efficiency.

Generation of drug-resistant effector and target cell lines by lentiviral transduction

The P140KMGMT cDNA sequence was inserted into the SIV expression vector by replacing the cDNA sequence encoding eGFP (Fig. 1A). Virus titers, determined using 293T cells as targets, were 107–108 TU/ml. Gene transfer into effector and target cells was quantitated by real-time PCR amplification using genomic DNA isolated from cells transduced with the recombinant virus at an MOI of 40. P140KMGMT copy numbers for the transduced NK-92, TALL-104 and K562 cells were determined to be 3 ± 0.28, 1 ± 0.14, and 4 ± 0.41, respectively. MGMT mRNA levels were also measured in K562 to confirm that gene-modified cells express increased levels of MGMT message. MGMT mRNA expression was readily detected in the transduced K562 cells while MGMT mRNA expression levels in untransduced K562 cells was below the linear range of detection, and therefore, the relative increase in mRNA levels was not possible to quantitate. Previous studies also reported extremely low MGMT protein levels in wild-type K562 cells [22].

To determine the effectiveness of the transferred P140KMGMT sequence, transduced and untransduced cells were incubated with 6-BG for 2 h. TMZ was then added at increasing concentrations and the cells were incubated for 48 h. Survival curves were generated with respect to the untreated cells. P140KMGMT transduced NK-92, TALL-104 and K562 cells were resistant to the 6-BG/TMZ combination when compared to untransduced control cells (Fig. 2A). Such resistance was pronounced up to 200 μM TMZ, where nearly all modified cells survived the drug challenge. The degrees of resistance achieved by genetic modification of each of the cell lines were measured by calculating the TMZ IC50 value, which was 360 ± 8 and 135 ± 4 μM for gene-modified and unmodified NK-92 cells; 385 ± 5 and 120 ± 6 μM for gene-modified and unmodified TALL-104 cells; and 550 ± 8 and 170 ± 10 μM for gene-modified and unmodified K562 cells, respectively. We therefore conclude

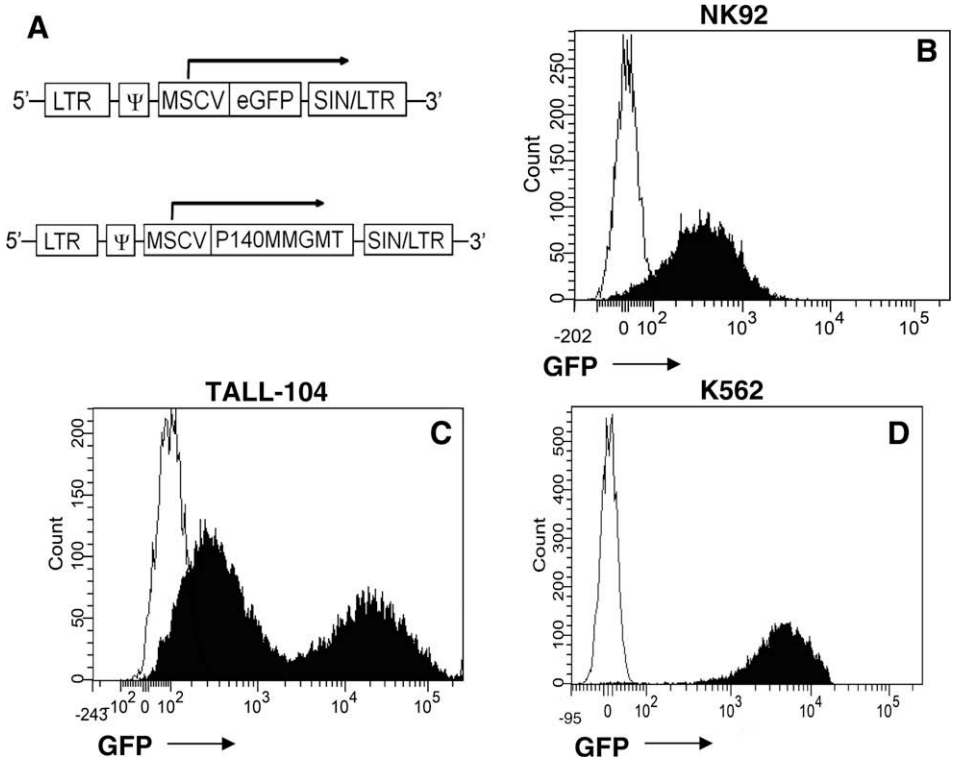

Fig. 1. Determination of transduction efficiencies for the various immunocompetent and target cell lines: schematics of SIV vector constructs encoding for eGFP and P140KMGMT (A). Flow cytometry analyses of NK-92 (B), TALL-104 (C) and K562 (D) cells transduced with SIV-eGFP lentivirus.

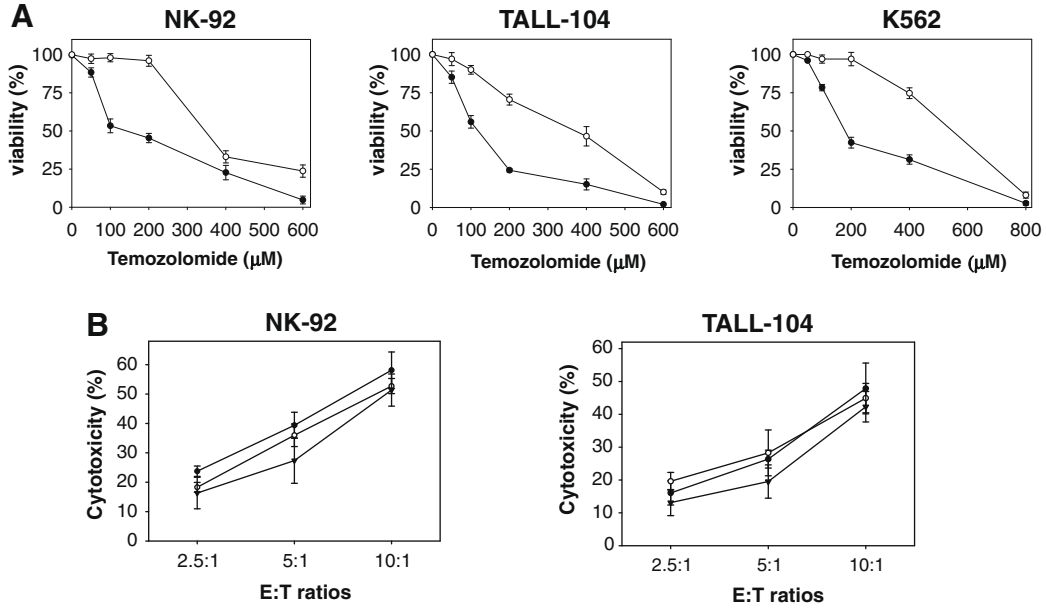

Fig. 2. Analysis of modified and non-modified effector and target cells: (A) survival curve analyses of P140KMGMT-modified (open circles) and non-modified (closed circles) NK-92 (left panel), TALL-104 (middle panel) and K562 (right panel) cells after 6-BG/TMZ treatments. The cells were treated with 25 μM 6-BG and increasing concentrations of TMZ. Forty-eight hours later, cell viabilities were measured by trypan blue method. Cytotoxic activities of the immune effectors, NK-92 (left panel) and TALL-104 (right panel) cells against the target cells, K562, at different effector:target (*E*:*T*) cell ratios (B). Increasing amounts of (i) P140KMGMT-modified cells (open circles), or (ii) modified cells after selection with 25 μM 6-BG/200 μM TMZ (reverse triangle) or (iii) non-modified cells (closed circles) were mixed with a fixed concentration of the target cells and LDH release assays were performed.

that each of the cell lines showed approximately 3-fold resistance to TMZ in a 48 h viability assay. Similar resistance levels have been achieved in hematopoietic stem cells and K562 cells, but using a 7–10 day survival assay [22]. Our choice of a 48 h assay was based on our downstream processing cytotoxic assays.

Drug-resistant variants of NK-92 and TALL-104 cell lines mediate effective target cell killing

It has been previously reported that NK-92 and TALL-104 can efficiently lyse the leukemic cell line, K562. Here we evaluated if

genetic modification of these immune-effector cells changed their cytotoxic abilities. Genetically engineered and non-modified effector cells were mixed with a fixed number of target cells at various effector:target ratios of 2.5:1, 5:1 and 10:1. Killing effectiveness of each of the drug-resistant effector cells was compared with the unmodified control cells in a 4 h cytotoxicity assay. When compared to the non-modified cells, both the gene-modified drug-resistant immune-effector cells, NK-92 and TALL-104 cells, showed similar cytolytic activities toward the target cell line (Fig. 2B).

We next determined if gene-modified immunocompetent cells retained their cytotoxic effectiveness after being expanded in the presence of 200 μM TMZ. As shown in Fig. 2B, the cytotoxicities of the drug selected gene-modified effector cells, NK-92 and TALL-104, were similar to the cytotoxicities of the non-selected gene-modified effector cells. These results show that the drug-resistant immunocompetent cell lines, after modification with P140KMGMT and selection with TMZ, retained their ability to efficiently lyse target cells.

Evaluation of drug-resistant immunotherapy

To test the concept of drug resistance immunotherapy, a series of combinatorial cytotoxicity assays were performed with non-modified and gene-modified effector and target cells. To determine the effects of TMZ on non-modified effector cells, cytotoxicity assays were performed whereby non-modified effector cells were mixed with gene-modified target cells in either the absence or presence of 6-BG/TMZ. Gene-modified target cells were used to eliminate the effects of chemotherapy on the target cells, which are resistant to 200 μM TMZ. Before these studies were initiated we also determined the sensitivity of gene-modified K562 cells to NK-92 and TALL-104 cells. A four hour cytotoxicity assay was conducted where non-modified effector cells (E) were incubated with either non-modified targets (T) or gene-modified target cells (T_m) at an effector to target ratio of 10:1 in the absence of drug (Fig. 3). We observed that the cytotoxicities of both the non-modified effector cell lines, NK-92 and TALL-104, toward either non-modified or gene-modified target cells were comparable ($P_{NK-92} = 0.8441$, $P_{TALL-104} = 0.6349$). Thus genetic modification of the target cells did not affect their lyses by the immunocompetent cell lines.

Cytotoxic assays were then conducted using non-modified effector cells and gene-modified target cells in the presence of TMZ. A significant decrease in both NK-92 and TALL-104 cell mediated lysis was observed compared to the lysis using gene-modified target cells in the absence of drug treatment (Fig. 3A and B; $P_{NK-92} = 0.0003$, $P_{TALL-104} = 0.0008$). Thus, the killing of drug-resistant tumor cells by non-modified immunocompetent cells is severely limited after a chemotherapy challenge.

To compare the killing effectiveness of non-modified and gene-modified immune-effector cells during drug treatments, cytotoxicity assays were conducted whereby non-modified or P140KMGMT-modified effector cells (E_m) were incubated with gene-modified target cells (T_m) in the presence of 6-BG/TMZ. When compared to non-modified effector cells, genetically-modified NK-92 cells lysed target cells significantly better after treatment with 6-BG/TMZ (Fig. 4A; $P_{NK-92} = 0.0001$). Thus, in the presence of drug, P140KMGMT-modified immunocompetent cells efficiently killed tumor cells. Under identical conditions, genetically engineered TALL-104 cells had only a modest increase in cytotoxic activity (Fig. 4B).

To determine the effectiveness of drug-resistant immunocompetent cells during a chemotherapy challenge, gene-modified effectors, i.e. P140KMGMT–NK-92 and P140KMGMT–TALL-104 cells, were incubated with non-modified, drug-sensitive target cells in the presence of 6-BG/TMZ. The cytotoxicities of these drug-resistant immunocompetent cells were then compared with the cytotoxicities achieved using drug-sensitive immunocompetent cells (Fig. 4C and D). Compared to the killing by non-modified effector cells, we observed a dramatic increase of nearly 4.5-fold and 2.5-fold killing of non-modified target cells by the genetically-modified NK-92 and TALL-104 cells (Fig. 4C and D; $P_{NK-92} = 0.0012$, $P_{TALL-104} = 0.0011$). These data demonstrate that P140KMGMT-modified NK-92 and TALL-104 cells function as potent effectors in the presence of 6-BG/TMZ and that drug-resistant immunocompetent cells, when used concurrently with chemotherapy, can significantly enhance the killing of target cells.

Discussion

A major limitation to chemotherapy treatments for cancer is drug induced immune toxicity. One strategy to combat the severe toxicity is to genetically engineer blood or marrow cells by the

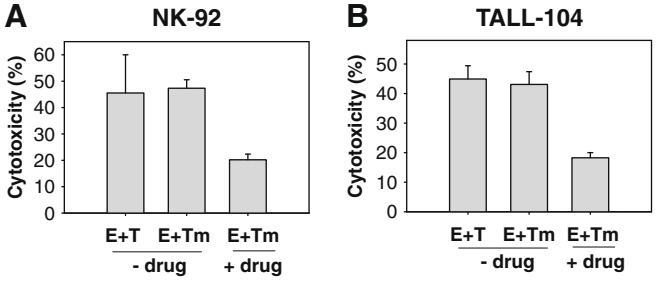

Fig. 3. Effective cell lysis of drug-resistant target cells: non-engineered effector cell mediated lysis of the target cells, K562, in the absence or presence of drug. The non-modified (E) effectors were incubated with either non-modified (T) or genetically-modified (T_m) target cells, K562, at an E:T ratio of 10:1 in the absence of drug. The non-modified effector cells along with the genetically-modified target cells were also treated with 25 μM 6-BG/200 μM TMZ overnight. These cells were then mixed at an E:T ratio of 10:1 and cytotoxicity assays were performed. (A) NK-92 cell mediated lysis and (B) TALL-104 cell mediated lysis.

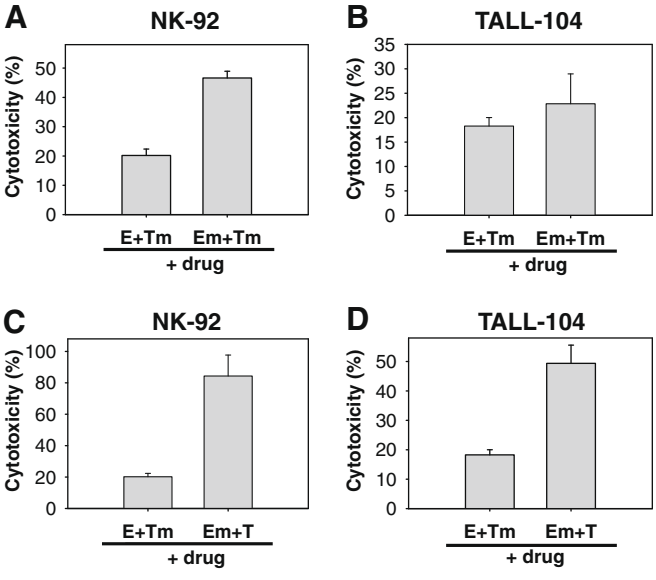

Fig. 4. Genetically engineered immune-effector cell mediated lysis of target cells: non-modified (E) and genetically-modified (E_m) effector cells along with the non-modified (T) or genetically-modified (T_m) target cells were treated with 25 μM 6-BG/200 μM TMZ overnight. (A and B) Either non-modified or modified effector cells were incubated with gene-modified target cells at an E:T ratio of 10:1 and cytotoxic activities of the effector cells were measured. (C and D) Combinations of non-modified or genetically-modified effector cells were mixed with either non-modified or genetically-modified target cells at an E:T ratio of 10:1, and cytotoxic activities of the effector cells were measured.

introduction of retroviral vectors designed to express cDNA sequences that confer drug resistance. Introduction of drug-resistant genes into hematopoietic stem cells (HSCs) results in transgene expression throughout the host hematopoietic system, including immunocompetent cells such as T cells and natural killer cells, after transplantation of gene-modified cells [20]. We hypothesized that expression of a cDNA sequence that confers drug resistance within immunocompetent cells would allow for the combined use of chemotherapy and immune-effector cell-based immunotherapy.

Previously our laboratory evaluated the feasibility of using drug-resistant immunotherapy in the context of drug-resistant hematopoietic cells [20]. Mouse bone marrow cells were genetically engineered by retroviral mediated introduction of a cDNA encoding for a mutant form of DHFR, L22Y–DHFR, that confers resistance to trimetrexate (TMTX). Mice were transplanted with gene-modified bone marrow cells, which resulted in transgene expression in all hematopoietic lineages, and treated with an immunotherapeutic agent, anti-CD137, or TMTX, or anti-CD137 and TMTX. In mice inoculated with AG104 sarcoma cells, TMTX chemotherapy reduced the efficacy of an anti-CD137 antibody in mice transplanted with non-modified cells. However, when mice were protected against chemotherapy-induced toxicity through transplantation of L22Y–DHFR-expressing bone marrow, the combined treatment of TMTX and anti-CD137 resulted in complete eradication of tumors in 100% of animals. In contrast to the protection of the entire hematopoietic system, in the present study we evaluated if genetically engineered human immunocompetent cells can be used in the context of drug resistance immunotherapy.

NK-92 and TALL-104 cells were selected as representative immune-effector cell lines since both of these cell types recognize and kill a wide range of malignant cells, including K562 cells [15,18]. We found that P140KMGMT genetically engineered NK-92 and TALL-104 cells were resistant to TMZ and had cytotoxic activities similar to the non-modified cells. Additionally, the gene-modified cells showed cytolytic activities similar to non-transduced cells after drug selection. Therefore, genetic modification of these cells, and likely other anti-tumor immunocompetent cells, does not affect their cytotoxic activity.

The concept of drug-resistant immunotherapy was then evaluated in a series of cytotoxic assays, in the presence and absence of drug. Importantly, gene-modified immunocompetent cells displayed significant cytolytic activities toward drug-resistant tumor cells in the presence of drug. In contrast, non-modified immunocompetent cells were ineffective at tumor killing when drug was administered. Taken together, these results demonstrate that in the presence of drug (i) the effectiveness of non-modified effector cells is significantly diminished, (ii) gene-modified effector cells remain active, and (iii) greater killing is observed after treating gene-modified effector cells and non-modified target cells compared to non-modified effector cells and drug-resistant target cells. Therefore, we conclude that genetically-modified drug-resistant immunocompetent cells can be engineered to survive the toxic effects of chemotherapeutic agents and the effectiveness of tumor killing increases during a chemotherapy challenge.

Our in vitro proof-of-concept studies demonstrate that drug-resistant immunocompetent effector cells are superior cytotoxic effectors during a chemotherapy challenge. This is a significant finding which can potentially be combined with current cell-based and adoptive immunotherapies. Regression of large, vascularized tumors has been shown in patients with refractory metastatic melanoma. However, for maximum effectiveness a lympho-depleting regimen is necessary prior to autologous lymphocyte cell transfer [23]. Generation and expansion of drug-resistant lymphocytes ex vivo can allow, in this setting, for administration of immunocompetent cell-based therapy concurrently with chemotherapy, potentially improving tumor clearance while anti-tumor immunity is established and maintained. In this scenario, non-transduced lymphocytes can continually be depleted using a selective chemotherapy treatment, which could be repeatedly applied during the administration of adoptive immunotherapy. The co-administration of chemo- and immunotherapies could then lead to long-term tumor clearance. However, it has also been shown that the growth of CML cells in mice transplanted with bone marrow engineered to confer resistance to MTX can be exacerbated by the administration of chemotherapy [24]. Thus chemotherapy treatment in the context of gene-modified whole bone marrow protection may induce secondary effects such as immune suppression that allow some cancers to survive a drug challenge. Based on our results, instead of transplanting drug-resistant hematopoietic stem cells, a more effective strategy could involve transplantation of drug-resistant immunocompetent lymphocytes.

It has recently been shown that melanoma and glioma cell lines are sensitive to the combination of TMZ and antifolates [25]. Retroviral transfer of dual vectors that co-express multiple cDNAs that confer resistance to multiple chemotherapy agents can be used to improve tumor cell killing by the administration of a combination of chemotherapeutic agents. For example, expression of DHFR mutants provide resistance to antifolates, such as MTX, while MGMT expression provides resistance to monofunctional methylating agents, such as TMZ. Furthermore, the knowledge of drug-resistant mechanisms for many chemotherapy agents can be employed to genetically engineer immunocompetent cells that are resistant to numerous anti-cancer agents. The engineered cells could then be tested in the context of drug resistance immunotherapy, which our results indicate should improve the effectiveness of tumor killing during a chemotherapy challenge.

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