Cytotoxic T Cells Reactive to an Immunodominant Leukemia-associated Antigen can be Specifically Primed and Expanded by Combining a Specific Priming Step With Nonspecific Large-scale Expansion

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Summary: Identification of dominant leukemia-associated neoantigens and favoring specific priming and subsequent expansion of T cells reactive to these antigens might harbor therapeutic potential. Here, a new strategy combines a specific T-cell activation step using tumor lysate-pulsed bone marrow-derived dendritic cells with a nonspecific large-scale expansion method. The leukemia cell line C1498 transduced with a potentially immunodominant antigen (ovalbumin) was used to track expansion and functionality of antigen-specific cytotoxic T cell (CTL), both in vitro and in vivo. Three times more leukemia-specific CTL could be generated when compared with the respective controls. CTL generated after increasing the antigen-specific T-cell precursor frequency in vitro cured up to 80% of mice bearing leukemia with the respective antigen ($P < 0.005$, as compared with controls). Alternatively, the yield of CTL reactive to a immunodominant neoantigen increased by factor 2 to 6 when T-cell donors were immunized with dendritic cell presenting the respective antigen. However, increasing the leukemia-reactive precursor frequency to a clinically exploitable level will be the key for the design of successful T-cell therapy trials.

Key Words: adoptive immunotherapy, CTL, leukemia, expander beads, dendritic cells, immunization, mouse

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Adoptive transfer of Ag-specific cytotoxic T cells (CTLs) has been increasingly recognized as an important therapeutic tool for certain human malignancies.[1] In 1982, Rosenberg and coworkers[1] demonstrated for the first time, that lymphocytes obtained from the peripheral blood of cancer patients mediated lytic activity against fresh tumor cells after nonspecific stimulation ex vivo.[2] However, in leukemia patients, with the exception of a subset of tumors, there is a general inability to isolate and expand T cells of therapeutic significance. Increasing knowledge about the existence of tumor-associated antigens has led to the attempts to generate tumor-specific T cells completely in vitro.

Characterization of peptides serving as specific targets for cytotoxic T lymphocytes have led to encouraging approaches including genetic modifications of T-cell receptors (TCRs).[3] These methods of cloning and expanding T cells can require several weeks to generate large number of cells as they are required for adoptive T-cell–based therapies.[4]

These limitations have been in part overcome using artificial microspheres coated with stimulatory and costimulatory molecules.[5] As activation of T cells is an important prerequisite for generating effective antileukemia responses, αCD3/αCD28-coated microspheres have been used for the ex vivo activation and consecutive expansion of CTLs for cancer immunotherapy.[6]

However, those techniques expand a rather nonspecific population of tumor-reactive CTLs. Hence, systems that specifically prime and expand a small number of cytotoxic precursor T-cells directed against different leukemia-associated antigens might harbor an important therapeutic potential.

Here, we investigated the capacity of αCD3/αCD28-coated microspheres to preferentially expand a CTL population that was generated by coculture of naive T cells with whole tumor lysate pulsed dendritic cell (DC). Using a genetically modified leukemia cell line expressing ovalbumin (OVA) as an immunodominant neoantigen, we provide proof of the concept that CTL directed against immunodominant antigens can be effectively expanded by combining a specific and physiologic priming strategy with a nonspecific but powerful expansion

system. The antileukemia effect mediated by the generated CTL was TCR-mediated and was specifically targeted against leukemia cells expressing the neoantigen. These data suggest that identifying immunodominant antigens and recognizing the respective CTL counterparts, specific priming of these CTL and their subsequent expansion would improve outcome, as the response would be more dominated by specific tumor-reactive CTL.

MATERIALS AND METHODS

Cell Lines and Mice

C1498, a C57/BL6 (H-2^b)-derived myeloid leukemia line expressing major histocompatibility complex (MHC) class-I has been previously described.[7] The murine cell lines EL4 (H-2^b) is a lymphoma line and BM185 (H-2^d) a lymphocytic leukemia line.[8] The cell lines were obtained from the American Type Culture Collections (ATCC) (Rockville, MD) and grown in a serum-free medium (AIM V—Gibco, Eggenstein, Germany). Full length OVA cDNA was cloned into the eukaryotic expression vector MMP-IRES-GFP.[9] Retroviral VSV-G-pseudo typed particles were produced by transient transfection of the producer cell line 293GPG with the MMP-OVA-IRES-GFP and MMP-IRES-GFP constructs and daily collection of the cellular supernatant over a period of 5 days. Viral titers were determined using 3T3 cells transduced with serial dilutions of the viral supernatant. For retroviral transduction of the C1498 cell line, 500 μL of the viral particles (5×10^6/mL) were added to 0.5×10^6 C1498 cells (in the presence of 8 μg/mL polybrene). The cells were incubated with the virus at 37°C for 1 hour, and then spin-inoculated at 37°C for 3 hours. After 4 days of culture, GFP-expressing cells were sorted and cultured to obtain the 2 new cell lines: C1498-OVA-IRES-GFP and C1498-IRES-GFP (referred to as $C1498_{OVA}$ and $C1498_{CONTROL}$). Single clones of the obtained cell lines were generated by limiting dilution assays. C57BL/6 ($H2^b$) (termed B6) mice, 6 to 12 weeks old at study, were obtained from Charles River, Germany. Transgenic OT-I mice ($OVA_{323-339}$ –SIINFEKL specific $H2^b$-restricted TCR-transgenic mice on C57/BL6 background) have been generously provided by B. Fleischer, Bernhard Nocht Institute Hamburg, Germany, and have been previously described.[10] All experiments were conducted under approved animal protocols of the state government of Lower Saxony, Germany.

Bone Marrow-derived DC Isolation and Antigen Pulsing

Bone marrow (BM) was harvested from the long bones of the femur, tibia, and fibula of mice as previously described.[11] Briefly, red cells were lysed by ammonium chloride incubation and the single cell suspension were then incubated at 2×10^6 cells/mL Iscove Modified Dulbecco Medium-complete media at 37°C and 5% CO_2 in 6-well plates with GM-CSF (Sigma, Munich, Germany) 150 U/mL and IL-4 (Sigma, Munich, Germany) 75 U/mL for 7 days. On day 8, DCs were pulsed with tumor lysate as described earlier.[12] This was followed by maturation of DC using CpG oligodeoxynucleotide as a maturing agent on day 9. The oligodeoxynucleotide sequence is TCG TCG TTT TTC GGT CGT TTT (CpG 10103, 2 μg/mL) (Coley Pharmaceutical Group, Germany). In some confirmatory experiments, supernatants of genetically engineered cell line EL4-GM-CSF and EL4-IL4 was used. After 24 hours of incubation, DCs were harvested, irradiated with 2500 cGy, washed, and used for T-cell priming. Splenocytes or EL4 were coated with the SIINFEKL peptide using established protocols.

Induction and Expansion of Leukemia Reactive CTL In Vitro

$C1498_{OVA}$ and $C1498_{CONTROL}$ were grown in AIM V, a serum-free medium. DC and CTL generation were performed in complete medium, consisting of RPMI 1640, respectively, supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg/mL streptomycin, 100 U/mL penicillin, 50 mg/mL gentamicin, 0.5 mg/mL Fungizone, and 0.05 mM 2-ME. Cytokines used were human IL-2 (AMGEN, Munich, Germany) and murine IL-7 (R&D systems, Bad Nauheim, Germany). B6 mouse BM-derived DC (1×10^5/mL), pulsed with either full $C1498_{OVA}$ or $C1498_{CONTROL}$ cell lysate, were added to responder splenocytes (1×10^6/mL originating from either OT-I mice or wild type B6 mice) in 24 tissue culture plates (Nunc) at a responder-to-stimulator ratio of 10:1. After 5 days (referred to as day 15), this cell product was further cultured in the presence of αCD3/αCD28 (PharMingen, Heidelberg, Germany) mAb-coated beads at a 3:1 bead to cell ratio. 20 IU/mL IL-2 (AMGEN) and 5 ng/mL IL-7 (R&D Systems) were added. αCD3/αCD28 mAb-coated beads (Dynal, Hamburg, Germany) were generated as previously described.[5] The αCD3/αCD28 beads were removed after 2 days of culture (referred to as day 17). Cells were then further expanded using IL-2 and IL-7 at the given concentrations for 4 or in some experiments 6 for more days (referred to as day 21 and day 23, respectively).

Flow Cytometry

Cells (10^6) were washed and incubated with α-FCR (CD16/CD32) (2.4G2) (PharMingen, Heidelberg, Germany) at 4°C for 10 minutes to block nonspecific binding of fluorochromes. The following directly conjugated antibodies (PharMingen) were incubated with CTLs at 4°C for 30 minutes: TCR Vα2-biotin, TCR Vβ5.1, 5.2-phycoerythrin (PE), Streptavidin-APC, CD8α-(Conjugated to fluorescein isothiocyanate, PE, PerCP), CD4-PE, CD25-PE, CD44-PE, and CD69-PE. OVA reactive CTLs were tracked using biotin-labeled Pro5 MHC Pentamer H2-K^b/SIINFEKL (ProImmune, Oxford, UK). For detection of intracellular interferon γ(IFNγ) in a sample of ongoing culture, Brefeldin A (Sigma-Aldrich, Steinheim, Germany) was added into the cell culture for 6 hours at a final concentration of

10 μg/million cells. Cells were then first stained with anti-CD8 mAb, permeabilized with FACSlyse (Becton-Dickinson), and stained with αIFN-γ-PE. Cells were acquired using the FACS Calibur (Becton-Dickinson, Heidelberg, Germany).

Adoptive Transfer of CTL

Leukemia was induced by intravenous injection of a 100% lethal cell dose (6×10^5 cells per mouse for $C1498_{CONTROL}$ and 12×10^5 cells for $C1498_{OVA}$) on day -1 via the lateral tail vein into fully immunocompetent B6 mice. CTLs were intravenously injected into the leukemia bearing animals on day 0 at a cell dose of 40×10^6 CTL per mouse. For the experiments in which leukemia was injected subcutaneously the same cell doses were used for the respective leukemia. For these experiments either $C1498_{OVA}$ or $C1498_{CONTROL}$ was given on day -1 and the generated CTL product was given 4 days later via the lateral tail vein. Response to treatment was documented by serial caliber measurements.

In Vitro CTL Killing Assay

Cytotoxic activity of the generated CTL product against C1498 and its variants was measured by Just Another Method (JAM) assay using [methyl-^{3}H] Thymidine to label targets.[13] CTLs were plated in eight 2-fold dilutions. E:T ratios were started at 50:1 using 3 replicate wells per dilution step. Percent-specific killing of C1498 target cells was calculated using the following formula: %-specific killing $= (S - E/S) \times 100$ where E is the experimentally retained DNA in the presence of CTL (in cpm) and S is the retained DNA in the absence of CTL (spontaneous). Data are presented as the mean percent-specific killing of triplicate samples (± SE) from a representative experiment.

In Vivo CTL Killing Assay

Cytotoxic activity and specificity of CTLs were determined using carboxyfluoresceindiacetate-succinimidyl-ester (CFSE)-based in vivo killing assay as previously described.[14] Mice were treated by injecting 40×10^6 effector cells IV. Untreated mice were used as controls. Target cells were then prepared using splenocytes from B6 mice. RBC-depleted splenocytes were pulsed with SIINFEKL peptide (1 ng/10^6) and incubated for 60 minutes at 37°C. Coated splenocytes were intensively labeled with high CFSE (Sigma, Munich, Germany) at a final concentration of 5 μM. As a control noncoated splenocytes were weakly labeled at a lower concentration of CFSE (0.5 μM). The coated and noncoated fractions were mixed 1:1 and then injected IV at 2×10^7 cells per treated or untreated mouse. Specific in vivo cytotoxicity was determined by flow cytometric analysis of the CFSE-positive cells within spleens that were harvested 4 hours later. The ratio r between $CFSE^{hi}$ and $CFSE^{lo}$ cells was calculated for determining the cytotoxicity of the effector cells. Cytotoxicity was calculated by the formula $(1 - r_{control}/r_{treated}) \times 100$.

Statistics

The Kaplan-Meier product-limit method was used to calculate survival rates. Differences between groups were determined using Log-rank statistics. P values ≤ 0.05 were considered to be significant.

RESULTS

Expansion System of Leukemia Reactive CTL In Vitro

BM-derived DC that had been pulsed with whole C1498 cell lysate were used to prime wild type B6 splenocytes in vitro. The specific priming step was followed by nonspecific expansion using αCD3/αCD28 beads, IL2, and IL7. Beads were removed after 2 days of expansion. After 4 more days of culture CTLs were expanded up to 150-fold (Fig. 1A). Phenotypic analysis revealed a population almost entirely composed of highly activated CTL ($CD8^+/CD25^+/CD44^+$) (Fig. 1B). The generated CTL mediated strong cytotoxic effects against C1498 leukemia cells in vitro (Fig. 1C). This was reflected by a 40% cure rate after adoptive transfer of the CTL in leukemia bearing mice (Fig. 1D) demonstrating a strong reactivity against a disseminated tumor such as leukemia. However, the capacity of the system to not only generate leukemia-reactive CTL but to expand specific CTL precursors remained to be investigated.

Specific Priming and Expansion of Antigen-reactive CTL Using Tumor Pulsed DC

To model the expression of an immunodominant antigen in C1498 cells, gene-expressing chicken ovalbumin (OVA) was introduced into the tumor cells. Retroviral vectors encoding OVA-IRES-GFP or IRES-GFP for the respective control cell line were used to transduce native C1498 cells as described above. The cells expressing GFP were sorted and consecutively tested for GFP (Fig. 2A) and intracellular ovalbumin (Fig. 2B) expression by flow cytometric analysis.

Next, we analyzed the capacity of $C1498_{OVA}$ leukemia cell lysate-pulsed DC (referred as $DC_{C1498OVA}$) to generate an OVA-specific CTL response against $C1498_{OVA}$ in vitro. Splenocytes obtained from OT-I mice were either primed on $DC_{C1498OVA}$ or DC that had been pulsed with cell lysate from $C1498_{CONTROL}$ (referred as $DC_{CONTROL}$). The OT-I TCR is specific for the OVA peptide SIINFEKL presented on H-$2k^b$. No cytokines were supplemented. Cell numbers were counted after trypan blue exclusion. After 5 days of coincubation the total yield of cells upon harvest was higher after priming on $DC_{C1498OVA}$ than after priming on $DC_{CONTROL}$ (Fig. 2C). Whereas naive OT-I splenocytes contain about 10% $CD8^+$ T cells, this cell fraction had more than doubled after priming on $DC_{C1498OVA}$ and the total number of IFN-γ producing $CD8^+$ T cells was increased 8-fold as compared with the control group (Fig. 2D). After a 5-day priming period on $DC_{C1498OVA}$, CTL-mediated cytotoxicity against $C1498_{OVA}$ was observed. This could be completely abrogated by treating the targets with MHC-I

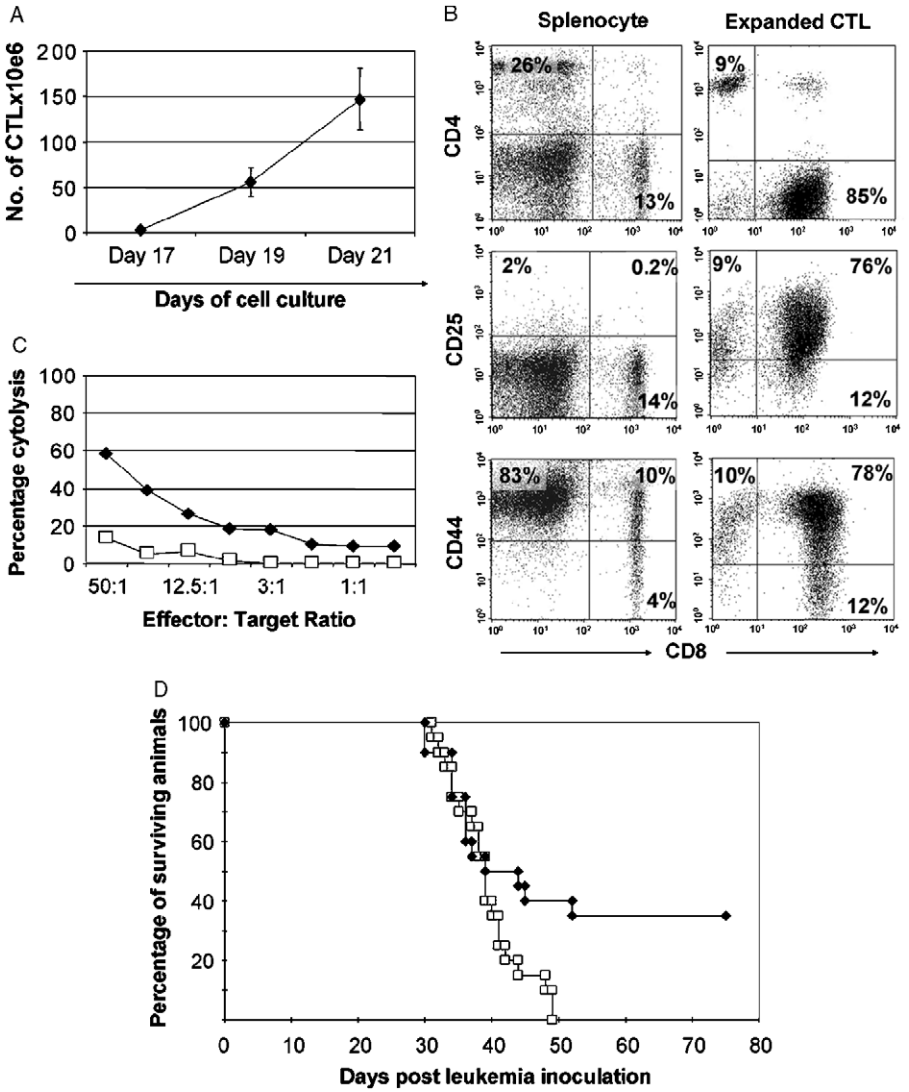

FIGURE 1. In vitro priming of splenocytes on DC_{C1498} followed by unspecific large-scale expansion generates CTLs reactive against C1498 in vitro and in vivo. BM-derived DC (days 0 to 10) were pulsed with C1498 cell lysates and subsequently used to prime splenocytes (days 10 to 15). Primed splenocytes were then expanded nonspecifically using anti-CD3/CD28-coated beads, IL2 and IL7 (days 15 to 17). A, Expansion of up to 150-fold was seen after 4 more days of culture (day 17 to 21). Values are shown ±1 SE. B, The cell product obtained after expansion consisted almost entirely of highly activated $CD8^+$ T cells as defined by expression of CD25 and CD44. Data represent one of 2 independent experiments. C, The expanded cell product was used in a JAM assay to quantify cytotoxic activity against C1498 cells. The expanded cell product (♦) mediated superior cytotoxicity as compared with naive splenocyte (□). D, Mice were intravenously challenged with a lethal dose of C1498 and subsequently divided in 2 groups. One group received 40×10^6 CTL by tail vein injection (♦) whereas the second group was treated with PBS only (□). Up to 40% of the mice could be rescued by the adoptive transfer of expanded CTL ($P<0.05$). Results shown represent pooled data from 2 independent experiments including 10 mice per group.

blocking antibodies. In contrast, when priming was done on $DC_{CONTROL}$ no significant MHC-I–dependent cytolytic activity against $C1498_{OVA}$ could be demonstrated (Fig. 2E). These data suggest that ex vivo pulsing of DC with whole-cell lysate derived from leukemia cells expressing an immunodominant antigen are able to prime specific T-cell precursors. However, total number of T cells remained relatively low.

As large scale ex vivo generation of T cells seems to be a prerequisite step for adoptive immunotherapy we asked the question whether leukemia-specific T cells could be further expanded after priming on $DC_{C1498OVA}$. Therefore, the preprimed T cells were further expanded in the presence of IL-2 and IL-7 using magnetic microspheres coated with αCD3 and αCD28. After 4 to 6 more days of expansion (day 19 to 23) the CD8:CD4 ratio and the overall percentage of T cells changed dramatically, with 97% expressing the CD8 coreceptor and less than 2% expressing the CD4 coreceptor. $CD8^+$ T cells upregulated CD25 (85% to 98%), CD 69 (27% to 30%), and CD44 (89% to 97%). CTL-mediated cytolysis against $C1498_{OVA}$ but not against $C1498_{CONTROL}$ or the irrelevant target, BM185 leukemia cells (Figs. 3A, B).

After subcutaneous (SC) injections C1498 cells form localized myelosarcoma like lesions. We used this model to quantify specific antileukemic activity of the adoptively transferred CTL product in the living animal. Mice received SC flank injections with $C1498_{OVA}$ cells into the left flank and $C1498_{CONTROL}$ cells into the right flank. Mice were then divided in a CTL treatment group and a phosphate-buffered saline (PBS) treatment group. Four days after the tumor challenge the treatment group received IV treatment with 40×10^6 in vitro generated OT-I–derived CTL. The second group was injected with PBS only. SC tumor growth is depicted in Figure 3C. The PBS group developed flank tumors bilaterally. In contrast, CTL-treated mice grew tumors on their right flanks only. Significance of these differences was assessed by pooling data from several experimental mice using caliper measurements ($P < 0.01$). This data strongly

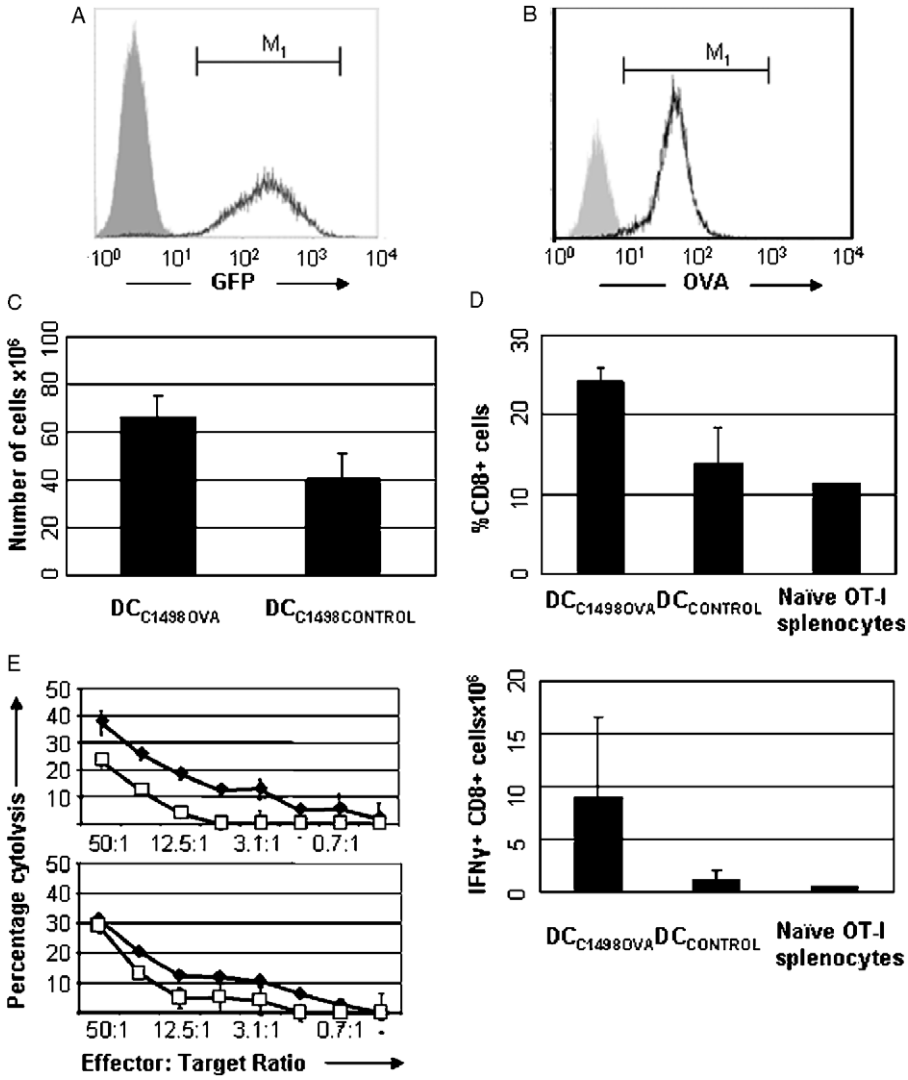

FIGURE 2. $DC_{C1498OVA}$ specifically primes ovalbumin reactive CTL. For retroviral transduction of C1498 leukemia cells, full-length ovalbumin cDNA was cloned into the eukaryotic expression vector cMMP-IRES-GFP. GFP expressing cells were sorted and cultured to obtain 2 new cell lines: $C1498_{OVA}$ and $C1498_{CONTROL}$. A, Expression of GFP in $C1498_{OVA}$ cells was determined by flow cytometry. B, Expression of ovalbumin by $C1498_{OVA}$ cells was demonstrated by flow cytometric analysis using intracytoplasmic staining. At least 10,000 events were analyzed for each sample. Data are presented as light line graphs for the expression of the respective proteins. The solid histogram graphs background fluorescence of the isotype control and M1 representing the positive gate. C, Total number of cells $CD8^+$ T cells is shown after priming OT-I–derived splenocytes on $DC_{C1498OVA}$ or $DC_{CONTROL}$ for a period of 5 days ($P<0.01$). D, Percentage of $CD8^+$ cells (upper panel) and the number of $IFN\gamma+CD8^+$ cells (lower panel) in the cell product obtained after priming OT-I–derived splenocytes on $DC_{C1498OVA}$ or $DC_{CONTROL}$ for a period of 5 days. Respective values in freshly isolated naive OT-I splenocytes are shown for comparison. E, After priming OT-I–derived splenocytes the resulting cell product was tested in a cytotoxicity assay using $C1498_{OVA}$ as targets. Cytotoxicity after priming on $DC_{C1498OVA}$ is shown on the upper panel and after priming on $DC_{CONTROL}$ on the lower panel, respectively. CTL-mediated killing was demonstrated by blocking MHC-I on the targets with $\alpha H\text{-}2K^b$ (□). For controls $\alpha H\text{-}2D^d$ (♦) was used. After priming with $DC_{C1498OVA}$ cytotoxic activity can be significantly ($P<0.05$) reduced by the addition of MHC-I blocking antibodies (upper panel), showing a CTL-mediated cytotoxicity. No significant difference was seen after MHC-I blocking when priming was done on $DC_{CONTROL}$ (lower panel). Very similar results were obtained in 2 independent experiments. Values are shown including ±1 SE.

suggests that the adoptively transferred CTL not only maintain in vivo activity after in vitro priming and expansion, but can also provide antileukemia effects specifically directed against a leukemia-associated antigen.

We and others have reported that migratory and homing characteristics of adoptively transferred CTLs are influenced by the generation modalities in vitro, which seemed to correlate with their antitumor therapeutic efficacy in vivo. This seems to have special importance in

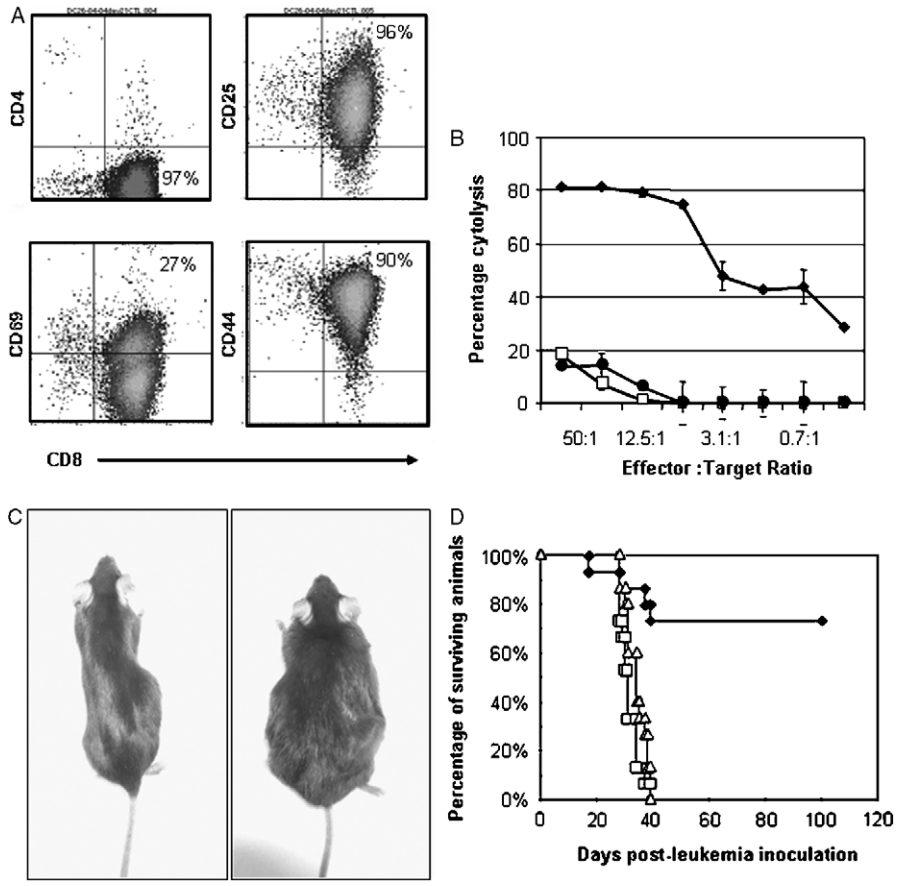

FIGURE 3. In vitro priming of OT-I–derived splenocytes on $DC_{C1498OVA}$ followed by unspecific large scale expansion results in a CTL population that mediates antigen-specific cytotoxicity in vitro and in vivo. $C1498_{OVA}$-reactive CTL were generated by coincubation of naive OT-I splenocytes with $DC_{C1498OVA}$ and further expanded for 5 days as described in the section Materials and Methods. A, The final cell product was phenotypically characterized by flow cytometric analysis. Data being representative of 3 independent experiments show an activated ($CD25^+$, $CD44^+$) CTL phenotype. B, In vitro testing for cytotoxicity demonstrates specific killing against $C1498_{OVA}$ (♦) but not against $C1498_{CONTROL}$ (□) and the irrelevant leukemia line BM185 (●) ($P<0.01$ against both controls). C, Mice were subcutaneously injected with 1×10^6 $C1498_{CONTROL}$ (right flank) and 1×10^6 $C1498_{OVA}$ (left flank) and then divided into 2 groups (n = 5 per group). One group was treated with adoptive transfer of OT-I–derived CTL 1 week later, the other group received PBS. After adoptive transfer of expanded OT-I CTL no tumor growth was observed at the site injected with $C1498_{OVA}$. In contrast, tumors developed at the contra lateral flank bearing $C1498_{CONTROL}$. Mice treated with PBS grew tumors bilaterally. D, Mice were intravenously challenged with a lethal dose of either $C1498_{OVA}$ (♦, Δ) or $C1498_{CONTROL}$ (□). $DC_{C1498OVA}$-primed and subsequently expanded OT-I CTL were adoptively transferred 1 day later into the leukemia bearing mice of both groups (♦, □). Higher survival rates were seen in mice challenged with $C1498_{OVA}$ and treated with expanded OT-I CTL as compared with $C1498_{CONTROL}$ ($P<0.05$). As a second control group $C1498_{OVA}$-challenged mice treated with PBS only were used (Δ). Differences between □ and Δ are statistically not significant. Data represent one of 2 independent experiments.

disseminated diseases such as leukemia.[15] Therefore, in subsequent experiments mice were intravenously challenged with a lethal dose of either $C1498_{OVA}$ or $C1498_{CONTROL}$. Two days later both groups of leukemia bearing mice received either 40×10^6 OT-I–derived CTL or PBS only. Whereas both, CTL-treated and PBS-treated $C1498_{CONTROL}$ bearing mice succumbed from leukemia 30 to 50 days after challenge, 80% of OT-I–treated $C1498_{OVA}$ bearing mice survived for at least 100 days postleukemia injection. Mice in PBS-treated control groups all died within 50 days (Fig. 3D). This demonstrates the specific antitumor effects of adoptively transferred CTL even in the context of a disseminated disease such as leukemia.

In Vitro Expansion of Antigen-specific CTL From a Polyclonal Population

Although conclusions drawn from results obtained in transgenic models are helpful in the context of proof-of-principle experiments, application and extrapolation from a TCR-transgenic to a polyclonal system can be problematic. Therefore, we tested the capacity of our in vitro CTL generation system to expand a subgroup of antigen-specific precursor T cells, which would mediate

specific cytotoxicity. Wild type B6 splenocytes were primed on $DC_{C1498OVA}$ or $DC_{CONTROL}$ and subsequently expanded as described. The resultant cell product was tested in a JAM assay using $C1498_{OVA}$ as target. Both, $DC_{C1498OVA}$-primed and $DC_{CONTROL}$-primed CTL-mediated cytotoxicity against $C1498_{OVA}$ (Fig. 4A), showing that the heterogeneous CTL population obtained was reactive against C1498-associated antigens. DC_{C1498}-primed CTL mediated similar killing against C1498 as $DC_{C1498OVA}$-primed CTL suggesting the presence of endogenous C1498-associated antigens (Fig. 4B). To rule out an adjuvant effect of the xenogeneic neoantigen OVA in vivo, wild type B6 mice were challenged with $C1498_{OVA}$ or $C1498_{CONTROL}$ (Fig. 4C). These mice were then treated with CTL primed with $DC_{CONTROL}$. Mice of both groups had a similar over all survival indicating that the antileukemia effect of the adoptively transferred CTL was not dependent on a

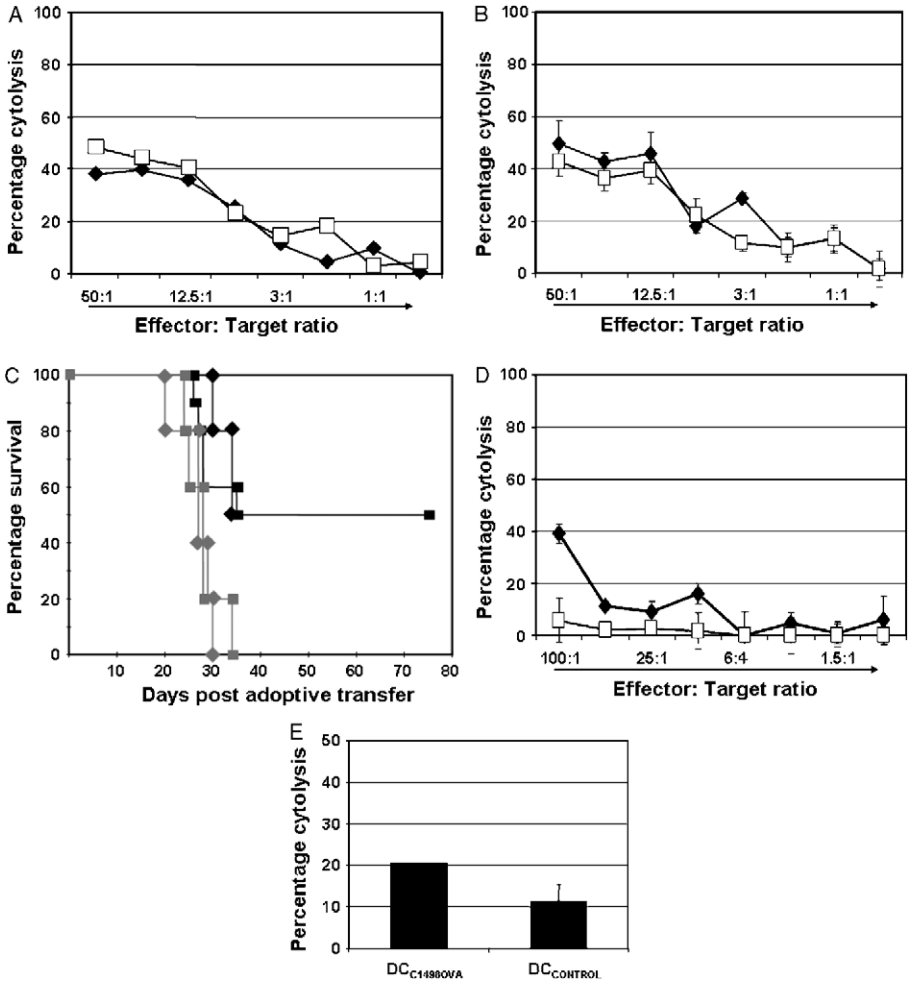

FIGURE 4. In vitro primed and expanded CTL-mediate specific cytotoxicity against immunodominant antigens in vitro and in vivo. Spleens from C57Bl/6 mice were primed on either $DC_{C1498OVA}$ (♦) or $DC_{CONTROL}$ (□). Primed splenocytes were subsequently expanded and tested for cytotoxicity against $C1498_{OVA}$ (A), $C1498_{CONTROL}$ (B), and EL4 coated with SIINFEKL peptide (D). $DC_{C1498OVA}$-primed and $DC_{CONTROL}$-primed CTL mediate comparable cytotoxicity against $C1498_{OVA}$ and $C1498_{CONTROL}$ suggesting a T-cell–mediated response against naturally occurring C1498-associated antigens (differences are not significant). However, only $DC_{C1498OVA}$-primed CTL mediate cytotoxicity against EL4 coated with SIINFEKL ($P = 0.003$). C, Wild type B6 mice were challenged with $C1498_{OVA}$ (diamond) or $C1498_{CONTROL}$ (square). One day later $DC_{CONTROL}$-primed CTLs were transferred into the leukemia-bearing mice (black). PBS-treated mice were used as controls (gray). Survival rates of CTL-treated mice and the PBS groups are significantly different ($P<0.01$). There are no significant differences between groups that were challenged with $C1498_{OVA}$ or $C1498_{CONTROL}$. E, $DC_{C1498OVA}$-primed or $DC_{CONTROL}$-primed CTLs were transferred into C57Bl/6 mice. Untreated mice were used as controls. Target cells were prepared by coating C57Bl/6 splenocytes with SIINFEKL and labeling them strongly with CFSE. Uncoated cells were labeled weaker with CFSE to allow separate identification of both populations by flow cytometric analysis. Both populations were mixed at a 1:1 ratio and injected as targets into the pretreated mice. After 4 hours, spleens were harvested and analyzed by flow cytometry for $CFSE^{hi}$ (SIINFEKL coated) and $CFSE^{lo}$ (uncoated) cells. Cytolysis was determined using the ratio r of $CFSE^{lo}$ and $CFSE^{hi}$ cells, respectively $[(1 - r_{control}/r_{treated})100]$. Mean cytolysis of 3 experiments is shown ($P<0.05$).

potential adjuvant effect mediated by the neoantigen OVA. As specificity control, EL4 lymphoma cells were coated with SIINFEKL peptide (Fig. 4D). Only $DC_{C1498OVA}$-primed CTL mediated cytotoxicity against SIINFEKL-coated EL4 indicating the presence of OVA-specific CTL after priming with $DC_{C1498OVA}$. However, these differences were only clear at the higher E:T ratios, indicating the relatively low frequency of OVA-specific CTLs. To test specificity in an in vivo setting, B6 mice were injected with 40×10^6 $DC_{C1498OVA}$-primed or $DC_{CONTROL}$-primed CTL as effectors cells. Untreated mice were used as controls. Splenocytes coated with SIINFEKL peptide were used as target cells after labeling with high concentrations of CFSE. Uncoated splenocytes were stained weaker with CFSE to control for nonspecific elimination. A 1:1 mixture of $CFSE^{hi}$ and $CFSE^{lo}$ cells was injected in the CTL-treated and control recipients. After 4 hours, splenocytes of the recipients were harvested and analyzed by flow cytometry for CFSE labeled cells (Fig. 4D). A lower ratio of $CFSE^{hi}$ to $CFSE^{lo}$ cells and therefore OVA-specific cytotoxicity was seen in mice treated with $DC_{C1498OVA}$-primed CTL as compared with those treated which $DC_{CONTROL}$-primed CTL confirming the generation of a CTL population that mediates specific in vivo cytotoxicity after adoptive transfer.

In our next experiments, we studied the expansion of an antigen-specific CTL population. As described previously, splenocytes were primed on $DC_{C1498OVA}$ or $DC_{CONTROL}$ and subsequently expanded. We tracked OVA-reactive CTL using the Vα2/Vβ5 TCR as a marker (Figs. 5A, B). The yield of antigen-reactive CTL could be increased up to 4-fold within 6 days of expansion. The specificity of the OVA-reactive population was confirmed using H2-K^b-SIINFEKL pentamers (Figs. 5C, D). The yield of OVA-specific CTL was 4-fold higher after priming on $DC_{C1498OVA}$.

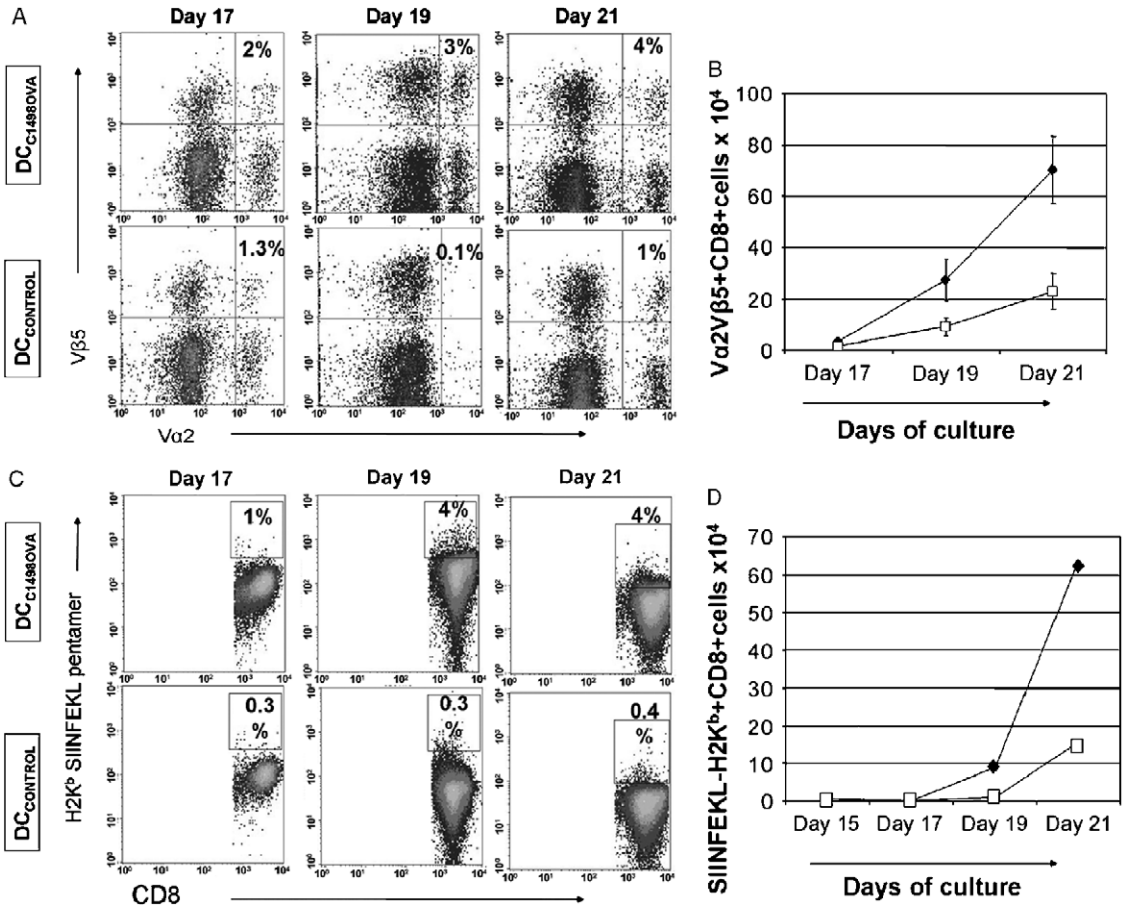

FIGURE 5. Preferential expansion of CTL reactive to an immunodominant neoantigen after priming on $DC_{C1498OVA}$. Splenocytes derived from wild type mice were either primed on $DC_{C1498OVA}$ or $DC_{CONTROL}$ (days 10 to 15) and subsequently expanded using αCD3/αCD28-coated beads (days 15 to 17). A, Live events gated on $CD8^+$ cells are shown. The upper plots in each group show the results after priming on $DC_{C1498OVA}$ and the lower plots on $DC_{CONTROL}$. B, The absolute number of Vα2/Vβ5 TCR+ $CD8^+$ cells is 3-fold higher after priming on $DC_{C1498OVA}$ (♦) as compared with $DC_{CONTROL}$ (□) ($P<0.05$). C, D, The OVA-specific CTL population was identified by SIINFEKL-H2K^b pentamer staining. The flow cytometric data and the absolute number of OVA-specific CTL reflect the data shown in panels A and B ($P<0.05$). The data shown are representative of 3 independent experiments.

Enhancing Dominant Antigen-specific CTL Cytotoxicity by Increasing the Precursor Frequency

To answer the question whether an increase of antigen-specific precursor T cells would result in a higher antigen-specific reactivity of the expanded cell product, OT-I splenocytes and wild type splenocytes were mixed at a ratio of 1:10, primed on $DC_{C1498OVA}$, and expanded subsequently. The resulting cell product was tested in a JAM assay using $C1498_{OVA}$ as targets. Cytotoxicity against $C1498_{OVA}$ was increased in vitro when naive splenocytes were "spiked" with antigen-specific precursor T cells before priming (Fig. 6A). We substantiated the findings in a CFSE-based in vivo cytotoxicity assay where spiked CTL effectors mounted stronger specific cytotoxicity against SIINFEKL-coated targets than CTL derived from "nonspiked" precursors (Fig. 6B).

Having demonstrated that an increase of antigen-specific precursor T cells gives rise to increased antigen-specific killing, we evaluated whether vaccination of naive B6 mice with $DC_{C1498OVA}$ would make them more efficient T-cell donors by increasing the OVA-specific precursor T cells. Control donors received vaccines with $DC_{CONTROL}$. As shown in Figure 6C, an increase of Vα2/Vβ5 expressing $CD8^+$ T cells as compared with the controls was seen in the draining lymph node. When splenocytes were reprimed with $DC_{C1498OVA}$ in vitro for 5 days and consecutively expanded as outlined above, the yield of OVA-reactive CTL was increased 2 to 6-fold as compared with the controls (Fig. 6D). This suggests that vaccination strategies of potential T-cell donors could be exploited to generate CTL products in vitro more efficiently.

DISCUSSION

This study suggests that CTL directed against an immunodominant leukemia-associated neoantigen can be generated in vitro by the combination of a specific activation step using BM-derived DC with an unspecific but effective expansion system consisting of αCD3/αCD28 mAb-coated microspheres. We have to emphasize at this point that studies using OVA-expressing tumors

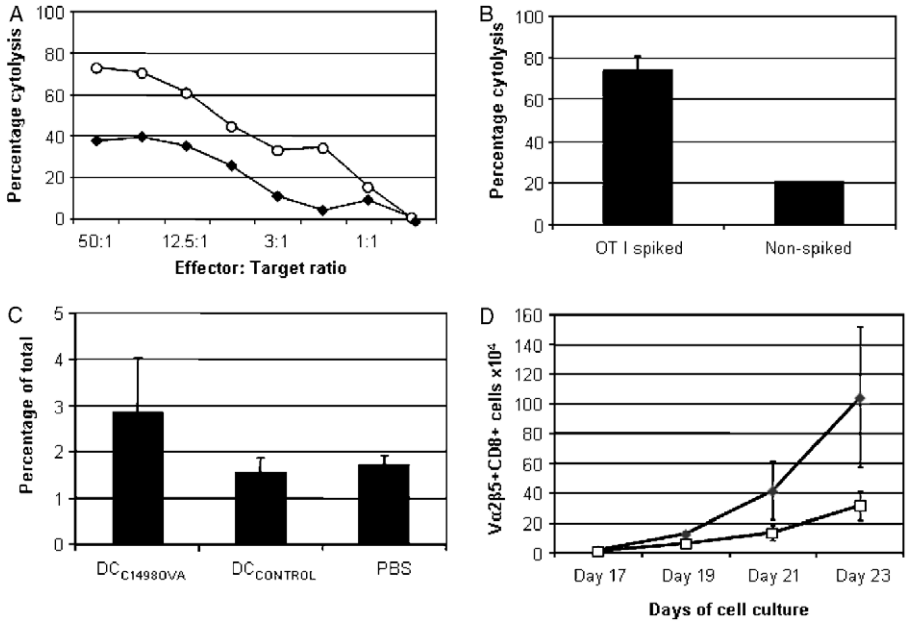

FIGURE 6. A higher frequency of OVA-reactive CTL precursors increases the yield of leukemia-reactive CTL. A, B, To increase the frequency of OVA-reactive CTL precursors, OT-I–derived splenocytes and wild type splenocytes were mixed at a ratio of 1:10. The spiked (O) splenocytes were primed on $DC_{C1498OVA}$, expanded, and then tested for in vitro cytotoxicity against $C1498_{OVA}$ (A). As compared with wild type, nonspiked splenocytes (♦), the cytolytic activity was about doubled against the OVA-expressing target ($P<0.05$). B, The spiked and nonspiked splenocytes were primed on $DC_{C1498OVA}$, expanded, and finally tested in a in vivo CFSE killing assay as described above. CTLs were transferred IV into C57Bl/6 mice. PBS-treated mice were used as controls. Target cells were prepared by coating C57Bl/6 splenocytes with SIINFEKL. Coated and noncoated cells were mixed at a 1:1 ratio and injected IV into the pretreated mice (2×10^7 cells per mouse). After 4 hours, splenocytes were analyzed as described above. Superior lysis of $CFSE^{hi}$ (SIINFEKL coated) targets is observed in mice treated with spiked CTL that were primed on $C1498_{OVA}$. Pooled data of 3 mice per group are shown ($P<0.01$). C, D, Wild type B6 mice were subcutaneously immunized with $DC_{C1498OVA}$, $DC_{CONTROL}$, or PBS. Seven days later draining lymph nodes (C) were harvested and analyzed for the presence of OVA-reactive Vα2/Vβ5+ $CD8^+$ cells. A 2-fold increase of Vα2/Vβ5+ CTL was seen in the draining lymph nodes. D, Splenocytes from $DC_{C1498OVA}$-(♦) or $DC_{CONTROL}$-(□) immunized mice were harvested, primed on $DC_{C1498OVA}$ (days 10 to 15), and subsequently expanded in vitro using αCD3/αCD28 beads (days 15 to 17). The resulting cell product contained a 2 to 6-fold higher yield of OVA-reactive CTL when splenocytes from $DC_{C1498OVA}$-immunized donors were used. Controls were immunized with $DC_{CONTROL}$ ($P<0.05$).

have significant limitations because a relationship to nonmodified tumors may be difficult to obtain. Nonetheless, our data shows that by skewing a T-cell response to a dominant tumor-associated antigen, in this case a xenoantigen, neoantigen, improved therapeutic efficacy can be obtained.

We sought to draw therapeutic advantage from the fact that antigen from "nonprofessional" antigen-presenting cell (APC) such as leukemia cells can be transferred to a "professional" APC such as DCs.[16] In a first set of pilot experiments we could show that $C1498_{OVA}$ can by itself function as a nonprofessional APC. Stimulation of naive OT-I–derived splenocytes with irradiated $C1498_{OVA}$ activated OT-I $CD8^+$ T cells that were able to lyse specifically $C1498_{OVA}$ but not $C1498_{CONTROL}$. We chose to use chicken OVA as a model tumor-associated antigen. Both, the immunodominant MHC class I binding peptide that elicits H-$2K^b$-restricted (SIINFEKL) and the ability of OVA-specific CTL to reject tumor cells that express OVA [OVA cDNA-transfected EL-4 (E.G7) cells] have been described.[17]

To transfer the leukemia-associated neoantigen to a professional APC we pulsed BM-derived DC with whole leukemia cell lysate derived from $C1498_{OVA}$. In practical terms, tumor lysate circumvents the need for viable fresh tumor cells and they avoid the necessity for molecular characterization of the tumor antigens. The advantage of this approach relates to the specific tracking of antitumor reactive T-cell responses on a clonotypic level for both number and function. As shown in Figure 1, the tumor lysates contain other unidentified leukemia antigens that provide a measure of T-cell immune response that is directed against bona fide (but unknown) leukemia-associated antigens as the consequence of CTL therapy does not result in elimination of all myeloid cells, just leukemia cells.[18]

Our data suggests that precursor CTL-specific against an immunodominant antigen can be specifically primed on BM-derived DC that have been pulsed with the respective cell lysate. As demonstrated by cell number and intracellular IFN-γ production preferential activation and expansion of TCR-transgenic leukemia-specific T-cell precursors within the $CD8^+$ compartment were achieved after 5 days of cocultivation with $C1498_{OVA}$ lysate-pulsed DC. Some unspecific activation was seen in the control groups as demonstrated by intracellular IFN-γ staining. However, we need to critically point out that only very limited numbers of antigen-specific CTL could be generated after 1 course of DC priming. Longer expansion systems can result in higher number of antigen-specific T cells, but this seems to be inevitably linked to a loss of critically needed homing capacity of these CTL after adoptive transfer.[16]

Although our studies indicate that anti-(acute myeloid leukemia) AML-reactive CTL can have a curative effect in a mouse model system, the challenges for extrapolation of these data to patients are substantial. It is important to keep in mind, that OVA was exclusively used as an immunodominant leukemia-associated surrogate antigen. This tool allowed specific identification, tracking, and functional in vitro and in vivo assays for specific T-cell–mediated immune responses. Using this tool we could demonstrate specific and preferential expansion of leukemia-reactive CTL. However, for the clinical approach T cells for CTL generation would need to be acquired from patients who have been exposed to AML cells and received prior chemotherapy. Such T cells may be dysfunctional and would need to be induced to become AML-reactive CTL. To overcome these limitations, several groups have set out to develop an approach to cancer immunotherapy on the basis of the genetic modification of normal peripheral blood lymphocytes. In first clinical trials, it could be demonstrated that normal autologous T lymphocytes, transduced ex vivo with antitumor–TCR genes and reinfused in cancer patients, can persist and express the transgene for a prolonged time in vivo and mediate the durable regression of large established tumors. These techniques seem be manageable once the respective good manufacturing practice-facilities have been established. Another concern certainly relates to the safety of retroviral vectors. A recent paper has addressed those issues. They analyzed the consequences of retroviral transduction in T cells from leukemic patients treated with allogeneic stem cell transplantation and donor lymphocytes genetically modified with a suicide gene (HSV-TK).[19,20] Given the therapeutic potential of gene transfer technology, there is no alternative to its testing in carefully designed clinical trials, where the risk-benefit balance must remain the central consideration.

In summary, if we can improve our understanding of tumor-associated immunodominant antigens, significant number of CTL against specific leukemia-associated antigens can be generated in a relatively short time span by combining a specific activation step with a more unspecific expansion strategy. However, innovative means to overcome the critical requirement of sufficient number of preexisting tumor-reactive cytotoxic precursor T cells are needed.

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