A Novel System for Simultaneous in Vivo Tracking and Biological Assessment of Leukemia Cells and ex Vivo Generated Leukemia-Reactive Cytotoxic T Cells

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ABSTRACT

To determine the mechanisms by which adoptive immunotherapy could reduce lethality to acute myelogenous leukemia (AML), a novel technique was developed to track both leukemic blasts and adoptively transferred cytotoxic T cells (CTLs) independently and simultaneously in mice. To follow the fate of ex vivo generated anti-AML-reactive CTLs, splenocytes obtained from enhanced green fluorescent protein transgenic mice were co-cultured with AML lysate-pulsed dendritic cells, which subsequently were expanded by exposure to anti-CD3/CD28 monoclonal antibody-coated magnetic microspheres. To track AML cells, stable transfectants of C1498 expressing DsRed2, a red fluorescent protein, were generated. Three factors related to CTLs correlated with disease-free survival: (a) CTL t-selectin expression. t-Selectin high fractions resulted in 70% disease-free survival, whereas t-selectin low-expressing CTLs resulted in only 30% disease-free survival. (b) Duration of ex vivo expansion (9 versus 16 days). Short-term expanded CTLs could be found at high frequency in lymphoid organs for longer than 4 weeks after transfer, whereas long-term expanded CTLs were cleared from the system after 2 weeks. Duration of expansion correlated inversely with t-selectin expression. (c) CTL dose. A higher dose (40 versus 5 x 10^6) resulted in superior disease-free survival. This survival advantage was achieved with short-term expanded CTLs only. The site of treatment failure was mainly the central nervous system where no CTLs could be identified at AML sites.

INTRODUCTION

The administration of escalating doses of multiagent chemotherapy in the treatment of acute myelogenous leukemia (AML) has significantly reduced the rate of relapse but is complicated by increased morbidity and mortality (1–6). Therefore, considerable interest has risen in alternative strategies such as the use of adoptive T-cell-based therapy that can target and lyse myeloid leukemic cells without the addition of toxicity to normal tissues (7). However, adoptively transferred cytotoxic T cells (CTLs) in some studies have been shown to have limited efficacy in part due to prolonged in vitro culture times that lead to loss of homing abilities and replicative senescence in the CTLs (8–10).

Imaging tools have been used to elucidate the pathophysiology governing tumor cell migration, adhesion, metastatic potential, and response to treatment. A major impediment improving cell therapy-based treatment approaches is an incomplete understanding of in vivo fate of both transferred CTLs and tumor cell target (11, 12). Elegant cell-tracking techniques have been developed over the last two decades such as bioluminescence imaging, radioactive labeling, magnetic resonance imaging, and positron emission tomography (13–16).

However, especially for disseminated malignant diseases such as leukemias, there is a paucity of studies tracking simultaneously and independently both tumor cells and adoptively transferred CTLs.

The expression of enhanced green fluorescent protein (eGFP) has been highly useful as a cell-tracking tool (17). New developments in imaging techniques and the availability of other fluorescent proteins, such as the red fluorescent proteinDsRed recently isolated from the Indo Pacific reef coral Discosoma species, now permit the simultaneous tracking of two cell populations in vivo with negligible cross-talk (18).

To examine the potential limitations of the adoptive transfer of anti-AML-reactive CTLs and to devise approaches that provide a superior anti-AML efficacy, we first generated a murine AML cell line that expresses DsRed2 and examined the metastatic patterns in mice that received various preparations of ex vivo expanded anti-AML-reactive CTLs derived from eGFP transgenic donors. Here, we show that adoptive CTL immunotherapy can result in long-term survival in up to 70% of mice, depending on the CTL product used for adoptive immunotherapy. Importantly, the curative effect of anti-AML-reactive CTLs was diminished as the culture time was increased. By infusing CTLs that expressed high levels of the lymphocyte homing receptor t-selectin, the highest fraction of mice was cured, suggesting that the migration to the secondary lymphoid organs was important for disease progression. This system of tracking CTLs and tumor cells should provide important new insights as to the limitations of adoptive immunotherapy applicable to other settings.

MATERIALS AND METHODS

Cell Lines and Mice. C1498, obtained from the American Type Culture Collection (Rockville, MD), was grown in AIM V, a serum-free medium (Life Technologies, Grand Island, NY; Ref. 19). Stable transfectants of the original cell line expressing the fluorescent Discosoma coral-derived proteinDsRed2 were prepared by cationic lipid delivery, cell sorting for fluorescence, and limiting dilution assays. The wells from which the cell lines were obtained contained 1 cell/well. The plasmid used for stable transfection contained both the Sleeping Beauty transposase (version 10) under cytomegalovirus promoter expression control and DsRed2 transposon under the control of the chicken β-actin promoter flanked by the transposase recognition sequences (20).

C57BL/6 (H2b) (termed B6) mice, 6–12 weeks old at study, were obtained from the NIH (Bethesda, MD). Transgenic B6 mice expressing eGFP have been reported (17). Cells that express eGFP can be detected by flow cytometry at a 488-nm emission wavelength. Mice were housed in microisolator cages under specific pathogen-free conditions. All experiments were conducted under approved protocols of the Division of Pediatrics at the University of Minnesota Cancer Center (Minneapolis, MN).

Bone Marrow (BM)-Derived Dendritic Cell (DC) Isolation and Antigen Pulsing. BM was harvested from the long bones of the femur, tibia, and fibula of mice as described previously (21). In brief, red cells were lysed by ammonium chloride incubation, and the single cell suspension was depleted of mature T cells, B cells, granulocytes, and IA^b (MHC II^b) cells by complement lysis. The following antibodies were used for depletion: anti-Thy 1.2 (30-H-12); anti-B220 (RA3–6B2); anti-Gr-1 (RA-8C5); and IA b (AF6–120.1.2; American Type Culture Collection). The DC progenitors were then incubated at 1 x 10^6 cells/ml DMEM-complete at 37°C and 10% CO2 in 6-well plates. Murine granulocyte macrophage colony-stimulating factor (150
In Vitro CTL Killing Assay. The cytolytic activity of the generated CTL population was measured using a 51Cr-release assay with K562 cells as targets. T cells were washed, resuspended to 2 × 10^6 cells/ml, and incubated with anti-CD4 (clone GK1.5; PharMingen) at a final concentration of 20 μg/ml to block nonspecific binding of fluorochromes. One or more of the following directly conjugated antibodies (PharMingen) were incubated with CTLs at 4°C for 10 min to block nonspecific binding of fluorochromes. One or more of the following directly conjugated antibodies (PharMingen) were incubated with CTLs at 4°C for 10 min:

- 5E2F/PE
- 14.4/PE
- 254/PE
- 255/PE

For detection of intracellular IFN-γ, CTLs were stained with anti-IFN-γ PE, permeabilized with FACSlyse (Becton-Dickinson) and stained with anti-IFN-γ PE and IL-2-PE (PharMingen). Cells were washed and analyzed using the FACScaliber (Becton-Dickinson) and were gated and analyzed using forward and side-scatter plots on 10,000 live events.

Statistical Analysis. 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

Spatiotemporal Tracking of C1498/DsRed2 Myeloid Leukemia Cells in Vivo. Stable transfectants of the C1498 leukemic cell line expressing DsRed2 were obtained after cell sorting for DsRed2 expression and limiting dilution, which permitted isolation of transfectants plated at 1 cell/well. The resulting 10 C1498/DsRed2 clones expressed the DsRed2 protein homogeneously but with different intensity. The two brightest clones were chosen and i.v. injected into B6 mice. Both were lethal at similar cell doses, which were about 2-fold higher than the uniformly lethal cell dose of the parental cell line (data not shown). One clone that maintained bright fluorescence in vivo was selected and passed three times through syngeneic recipients to maintain the cell line used for our experiments. After the injection of 1.0 × 10^6 C1498/DsRed2 cells, C1498 leukemic cells could be detected infiltrating the bone marrow, liver, spleen, lymph nodes (LNs), kidney, ovary, skin, and central nervous system (CNS), which are sites of AML disease in patients. In contrast, leukemia cells were not detected in the lung, which is not a frequent site of metastasis in AML patients. All recipients showed leukemic engraftment. In a first series of experiments, animals were sacrificed weekly, and the mice were exposed for single organ imaging. Although repetitive macroscopic imaging could not demonstrate fluorescent signals originating from C1498 leukemia cells during the first 18–21 days after injection, this time period, fluorescent leukemia deposits were readily detectable in the liver, LNs, colon wall, kidneys, and brain (Fig. 1). Twenty-one to 28 days after injection, DsRed2 fluorescence emitted from C1498 deposits could be detected through the abdominal wall over liver and intestines in living animals (Fig. 1A). From this time point, the leukemic burden progressed rapidly, and the animals died within the next 10–14 days.

Short-Term Expanded C1498-Reactive CTLs Control C1498 Infiltration into Lymphoid Organs but Fail to Migrate to or Expand in Nonlymphoid Organs. To generate anti-AML-reactive CTLs that could be tracked in vivo, splenocytes from eGFP donors
were incubated with AML lysate-pulsed DCs. CTLs were consecu-
tively expanded by exposure to anti-CD3/CD28 mAb-coated mag-
etic microspheres as described above. Before adoptive transfer into
C1498-bearing B6 mice, lytic activity was assessed in vitro using a
murine B6 melanoma cell line as an irrelevant target. To study the
potential interaction between adoptively transferred CTLs and the
AML cell line C1498 in vivo, 1 × 10^6 DsRed2-transduced C1498 cells were
injected into naive B6 mice. One day later, 5 × 10^6 anti-C1498-
reactive CTLs generated by short-term (9 days) expansion from GFP
mice were injected into the leukemia-bearing animals. A control
group of leukemia-bearing mice received no treatment. Cohorts were
analyzed for survival. Representative mice of each cohort were sac-
crificed 1, 3, 5, 7, 14, 21, and 28 days after adoptive T-cell transfer, and
organ systems were studied by confocal microscopy and fluorescence
imaging. Although treatment and control groups died from leukemia
at a comparable rate (Fig. 2A) after CTL injection, the development of
leukemia cell deposits and infiltrates was different between controls
and groups that received CTLs. Untreated mice developed leukemic
infiltrates in inguinal and axillary LNs that emitted strong fluorescent
signals transcutaneously in the living animal (Fig. 3A). In treated
mice, no leukemic infiltration of the LNs could be detected transcu-
taneously. Although extensive infiltration of C1498 in the LNs was
prevented in the treated animals, they eventually succumbed from
leukemia infiltrating nonlymphoid organ systems such as the CNS,
liver, and the intestinal tract. However, adoptively transferred CTLs
could be identified in LNs being in close cell-to-cell contact with
leukemic cells showing a vanishing DsRed2 signal (Fig. 3F). Whereas
CTLs could be readily identified in LNs and spleens, few could be
detected in the nonlymphoid organs. Thus, adoptive transfer of a low
dose of anti-C1498-reactive CTLs seemed to be effective in reducing
leukemia infiltrates in lymphoid but not nonlymphoid organs.

**Failure to Control Leukemic Growth in Peripheral Organs Can in Part Be Overcome by a Higher Cell Dose of Short-Term but Not Long-Term Expanded CTLs.** Finding no overall survival advantage for animals treated with adoptively transferred CTLs but evidence of interaction between the leukemia-reactive T cells and C1498 cells in vivo, we asked whether a higher dose of anti-C1498-
reactive T cells would result in a better treatment effect. To generate
high T-cell numbers for adoptive transfer, we used either larger
splenocyte numbers for seeding or extended the culture duration.
Whereas the CD8\(^+\) T-cell population was expanded by 50–100-fold
during short-term expansion, extension of the culture for another 7
days resulted in a total of 1000–5000-fold expansion (Fig. 4A). Cells
harvested during the expansion process and tested for their cytolytic
activity demonstrated preferential cytolysis against C1498 compared
with an irrelevant target, B16 melanoma cells (Fig. 4, B and C). Mice

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*Fig. 1. Metastatic sites of DsRed2-transfected AML cells. B6 mice received i.v.
injections of 1 × 10^6 DsRed2-transduced C1498 cells. Four weeks after injection,
fluorescent signals could be visualized transcutaneously. A, the lateral view of two
anesthetized mice with elevated forelegs that touch each other in the midline (marked by
the *vertical white line* separating the two halves of the figure). The heads and the hind
limbs are not included, leaving just the view of the lateral trunk. A leukemia-bearing
mouse was placed on the right and a healthy control animal on the left. Arrows (from top
to bottom) are pointing at DsRed signals originating from the axillary LNs, liver,
mesenterium, and ovaries. B, in the open abdomen of a sacrificed mouse, liver metastasis
is visible. At 3 weeks after injection, leukemic blasts can be identified in multiple organs
(e.g., colon) by confocal microscopy. C, leukemia cell deposits of 100–200 cells can
be easily detected within the colon wall. D, DsRed2-leukemia cells migrate to and can be
identified in the CNS. Images originating from one of three representative experiments
are shown.*

*Fig. 2. In vitro generated C1498-reactive T cells mediate antileukemia effects in vivo
after adoptive transfer. Unfractionated T cells were injected into C1498-bearing mice after
a shorter-term (day 9; □) or after a longer-term (day 16; ▪) culture period. Data are
presented in Kaplan-Meier curves. Both cell products were tested at dose levels of 5 × 10^6
T cells/mouse (A) and at 40 × 10^6 T cells/mouse (B). Differences in survival were not
statistically different from the untreated controls (●) at the lower T-cell dose level (A).
At the higher dose level (B) treatment with shorter-term cultured T cells resulted in signif-
icantly (*P < 0.05*) better survival rates when compared with untreated controls. In
contrast, adoptive transfer of longer-term cultured T cells did not increase overall survival.
A clear T-cell dose-response effect could be demonstrated for the shorter-term cultured T
cells only. Pooled results from two independent experiments are shown. Ten mice/group
were used in each experiment.*
challenged with a lethal dose of C1498 cells on day −1 were treated the next day with anti-C1498-reactive T cells, harvested after a shorter- or longer-term expansion period. For each cell product, two treatment doses (5 or 40 × 10^6 cells/mouse) were chosen. As expected from previous experiments, there was no survival advantage in the group treated with 5 × 10^6 T cells. However, when 40 × 10^6 of short-term expanded T cells were injected, up to 40% of the animals could be rescued. Interestingly, no significant survival benefit could be achieved after treating the animals with the same high-cell dose when CTLs were generated using the longer expansion process (Fig. 2B). Animals that received longer-term cultured T cells at a dose of 40 × 10^6/mouse developed C1498 infiltrates in the identical anatomical sites within the same time frame as the untreated control mice.

Duration of the ex Vivo Expansion Alters the Phenotype and Activation Status of Anti-AML-Reactive CTLs. To characterize the generated anti-C1498-reactive T-cell product, flow cytometry analysis was performed at three different time points during the ex vivo priming and expansion process (Fig. 5). After 5 days of coculture with C1498 lysate-pulsed DCs, the percentage of T cells was similar to that found in RBC-depleted naive splenocytes. The CD8:CD4 ratio was approximately 1:1 compared with a ratio of 1:2 in naive splenocytes. In contrast to a naive splenocyte population, CD25, CD44, and CD69 were up-regulated, and the T cells had a blastic appearance. After stimulation of the entire cell product with anti-CD3/CD28 mAb-coated magnetic beads and short- term expansion, the CD8:CD4 ratio and the overall percentage of T cells changed dramatically, with 85% expressing the CD8 coreceptor and up to 10% expressing the CD4 coreceptor. CD8^+ T cells up-regulated CD25 (50–70%), CD44 (40–60%), and CD44 (85–95%). Intracellular cytokine expression was at its highest with 10–20% of CD8^+ cells producing detectable levels of IFN-γ and 5–10% of CD8^+ T cells and about 50% of CD4^+ cells.
What was seen in CTLs generated from wild-type mice (data not shown). Anti-C1498-reactive T cells were injected into cohorts of C1498-bearing mice, and mice were sacrificed at the time points mentioned above. The mice were macroscopically searched for green fluorescent signals, and organs were harvested and processed for confocal microscopy examination. Additionally, LNs and spleens were processed for flow cytometric analysis to quantify GFP-positive cells. One day after injection, strong fluorescent signals could be detected in the spleen. Approximately 40–50% of the injected T cells could be identified in the spleen by flow cytometry analysis of mice that received short-term expanded CTLs. Three to 5 days later, the first GFP-positive cells could be identified in the inguinal and mesenteric LNs, reaching the highest frequency on day 7. GFP/CD8 double-positive cells were identified until the end of the observation period (day 28) at a frequency averaging a total of 30–40% of the originally injected CTL dose. In contrast, only 10–15% of the injected cells could be identified in the spleen after transfer of longer-term expanded CTLs. GFP/CD8 T cells obtained from spleen and LNs of recipients of either shorter- or longer-term expansion cultures were L-selectin⁺. Of note, already between day 7 and 14 post transfer, all GFP/CD8 double-positive signals disappeared from LNs and spleens of mice that had received long-term expanded CTLs (Fig. 6).

Differences in L-selectin expression between shorter-term and longer-term expanded CTLs were prominent. Because mice treated with longer-term expanded CTLs developed leukemic infiltrations in the lymphoid organs to a similar extent as untreated controls and others have proposed that L-selectin as an important homing receptor on T cells may serve as a surrogate marker for identifying tumor-reactive effector cells (27, 28), we fractionated shorter-term cultured CTLs according to their L-selectin expression in an attempt to improve cure rates. A purity of more than 95% was achieved as documented by flow cytometry analysis before adoptive transfer into leukemia-bearing animals. Before this fractionation, the generated cell product was depleted of CD4⁺ T cells to exclude the possibility that the different effects of the shorter- versus longer-term cultures on anti-C1498 leukemia effects was due to the lower content of CD4⁺ T cells in the latter, which may help support in vivo CTL persistence. Mice that received unfraccionated, L-selectin high, or L-selectin low or negative CTLs had a significant survival benefit compared with controls that did not receive CTLs (Fig. 7E). However, mice that received L-selectin high CD8⁺ T cells (40 × 10⁶) had a significantly superior survival compared with those that received L-selectin low or negative CD8⁺ T cells (70% vs. 30%, respectively; P < 0.05). Mice that received unfraccionated CD8⁺ T cells, containing both L-selectin high and L-selectin low or negative CD8⁺ T cells, had an intermediate long-term survival rate of 50%. In vivo homing of L-selectin high versus low or negative CTL preparations differed. Whereas L-selectin high T cells migrated readily into the lymphoid follicles of spleen and LNs 1 day after transfer, L-selectin low or negative T cells did so to a much lesser extent (Fig. 7, A–D). Treatment failure in the group treated with L-selectin high-expressing T cells occurred mainly because of CNS disease. Adoptively transferred CTLs could be easily identified in all of the studied organ systems except in the CNS, in which no transferred T cells were identified at any time point. In contrast, mice dying after treatment with the L-selectin⁻ fraction had disseminated leukemia.

**DISCUSSION**

We present a new system that allows assessment of in vivo migration and homing patterns of adoptively transferred leukemia-reactive CTLs and simultaneous and independent documentation of their interaction with leukemic cells systemically within the animal. To
render AML blasts traceable in the animal, cells were transfected to express DsRed (18). DsRed fluorescence is excited optimally at 558 nm but can also be excited by a standard 488-nm laser, allowing DsRed to be used with laser-based confocal microscopes and flow cytometers (29). As a source of syngeneic T cells, splenocytes were obtained from B6 mice transgenic for eGFP protein expression. This system allowed us to gain the following insights: (a) In vitro cultured T cells with anti-C1498 activity could be identified in the lymphoid organs of immunocompetent hosts for at least 28 days after adoptive transfer. (b) Phenotypic changes of T cells evolving during ex vivo expansion influenced the in vivo homing and migration of anti-C1498-reactive T cells. (c) Transferred T cells migrated to, interacted with, and eradicated C1498 leukemia cells under certain conditions in vivo. Our results also demonstrate that the level of l-selectin expression was an important determinant of the antileukemic effects achieved in vivo. High l-selectin expression resulted in a rapid migration of adoptively transferred T cells into peripheral lymphoid organs, which was associated with leukemic cell eradication in these organs, whereas l-selectin low/negative fractions resulted in a lower survival and homing into peripheral lymphoid organs. Leukemia progressed in organs (e.g., CNS) in which few transferred T cells were present.

Over the last years, different technologies for imaging cellular changes within animals have advanced to a point where it is possible to reveal the migratory paths of lymphocyte populations (30–32). One such technique, bioluminescent imaging, has been used in several very elegant studies over the past years for sequentially analyzing live animals (16, 33–35). In immunotherapy models either tumor or immune cells have been engineered to express the firefly luciferase gene enabling investigators to track one cell population or the other and provide information as to the number of cells present by quantifying signal intensity. Despite the numerous advantages of bioluminescent imaging, localization of cells within organs has been somewhat more difficult to ascertain as has the identification of the specific cell types that express luciferase in a given site. More importantly, the simul-
taneous tracking of two populations by bioluminescent imaging is not currently feasible. The techniques to simultaneously track two independent cell populations developed and applied in this study can provide important insights into the mechanisms of adoptive immunotherapy. In earlier reports by other investigators using solid tumor models, it was demonstrated that the expression of low rather than high levels of L-selectin on adoptively transferred T cells was advantageous for tumor-bearing animal survival. The infusion of L-selectin low T cells isolated from the draining LN of the tumor bed and expanded in vitro with anti-CD3 mAb + IL-2 migrated to and infiltrated within peripheral organs after transfer, resulting in the rejection of established tumors at that site (28, 36, 37). Based on these studies and on our results regarding the sites of metastases after C1498 infusion in nontreated mice or recipients of low T-cell doses, we had hypothesized that T cells harvested after a longer expansion period might be advantageous over shorter-term cultured T cells because the former would migrate readily into the nonlymphoid organs and prevent widespread metastases. This was not the case. In contrast, longer-term expanded CTLs were rapidly cleared from the organism and resulted in significantly inferior treatment effects as compared with the control group. Therefore, we considered the alternative hypothesis that early trafficking to lymphoid organs would prevent the subsequent seeding of nonlymphoid organs. In that event, the infusion of T cells selected for L-selectin high expression and preferentially migrating to secondary lymphoid organs may be advantageous. Consistent with this latter hypothesis, mice treated with the L-selectin high fraction had a significantly better survival than those that received the L-selectin low/negative fraction. Because L-selectin expression is lower in longer- versus shorter-term cultures, we hypothesize that this finding may account for the inferior antileukemia effect observed with longer-term cultures. To test this hypothesis, future studies will be required to compare the antileukemia efficacy of L-selectin high cells isolated from shorter- versus longer-term cultures. Nonetheless, together these data suggest that it will be important to consider the desired sites of T-cell homing to provide the most vigorous anti-tumor effects for a given tumor model or disease. Because L-selectin expression was down-regulated in longer-term cultured cells, our data would indicate that higher T-cell numbers were generated at the expense of homing to the sites of AML cells. Ex vivo propagated and transferred CTLs localizing within the LNs expressed high levels of L-selectin at all time periods after transfer, consistent with known homing and migration of L-selectin expressing T cells (38). Such observations were greatly facilitated by the use of the imaging technique that we described recently (25) and in this report. Of note, 40–60% of shorter-term expanded T cells were found in the spleen 1 d post-transfer, whereas only 10–15% could be identified there when T cells were harvested after a more prolonged expansion process. Although ICAM-1 (CD54) expression has been associated with T-cell homing to the spleen (39, 40), there was no difference in the proportion of cells or antigen density on cells between these two fractions. Because L-selectin expression is not required for lymphocyte homing to the spleen, receptors other than L-selectin or ICAM-1 (e.g., adhesion molecules, chemokine receptors, adrenergics, or sphingosine-1 phosphate receptor) that are known to regulate T-cell recirculation and homing to lymphoid organs other than LN may be differentially expressed on L-selectin high versus L-selectin low or negative cells (41). Additional studies beyond the scope of this report will be required to identify which of these account for the preferential homing of L-selectin high versus low/negative CD8+ T cells to the spleen.

Another striking finding in our study was the fact that no adoptively transferred CTLs could be identified in the CNS. Although the duration of the CTL ex vivo expansion process, the degree of L-selectin expression, and the cell dose infused significantly altered overall survival, migration of transferred CTLs into the CNS was not observed, and leukemic growth as easily identifiable by strong DsRed signals in the CNS suggesting that CNS disease was a major reason for treatment failure. This was unexpected because previous work has shown that the structure of the blood-brain barrier allowed T-cell trafficking of naive, activated, and memory/effector T cells to the CNS (42–44). However, to our knowledge, very little data exist regarding the migratory properties of ex vivo generated CTLs into the CNS. There is evidence that adoptively transferred T-lymphoblasts reach their peak concentration in the CNS between 9 and 12 h after transfusion and exit the CNS within 1–2 days (43). In our study, the earliest time the CNS was examined for the presence of transferred CTLs was 24 h after infusion. A relatively narrow time window might have been missed. Furthermore, recent experiences in clinical trials using EBV-specific CTLs for the treatment of EBV-associated lymphomas involving the brain and locations outside of the CNS have shown that the response to adoptively transferred CTLs is more vigorous in non-CNS sites (45). Together with our observations, this suggests that the CNS differs in a fundamental aspect from peripheral organs, which allow ready access and/or long persistence of adoptively transferred CTLs.

Although our studies indicate that anti-AML-reactive CTLs can have a curative effect in a mouse model system, the challenges for extrapolation of these data to patients are substantial. T cells for CTL generation would need to be acquired from patients that 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 CTLs. Nonmanipulated AML cells have been shown to be poor APCs, although cytokines or fusions with DCs have been used to support CTL generation from normal donors (7, 46, 47). Alternatively, the approach described here, in which DCs are loaded with AML lysates, would require sufficient AML cells that express immunogenic antigens or peptides to support CTL generation. Proof-in-principle does exist for this approach as reported by Galea-Lauri et al. (48), who demonstrated strong autologous anti-AML-reactive CTL generation against AML lysate-pulsed autologous DCs obtained from an AML-M2 patient in remission. Although there are not abundant examples of such CTL generation in AML patients, the opportunity exists for the application of such approaches for the generation of CTLs that can be expanded by anti-CD3/CD28 mAb-coated microspheres that already have been used in clinical trials (49). Using normal rodent splenocytes, such leukemia-reactive T cells could be expanded 50–5000-fold (depending on the duration of the expansion process) after exposure to anti-CD3/CD28 mAb-coated microspheres, a procedure that would provide both a T cell receptor and costimulatory signal and prevent or perhaps even reverse antigen-specific hyporesponsiveness (50).

In summary, we have established a tracking system for adoptive immunotherapy of AML. An imaging system could be developed that allows the investigator to visualize adoptively transferred T cells and leukemic cells independently in the same animal. This opens the opportunity to directly assess the effects of modifications during the in vitro CTL generation on their in vivo characteristics, which should lead to more optimized CTL therapy based upon analysis of homing and migration patterns of anti-leukemia-reactive CTLs.

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