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In vitro generation of tumor specific T cells that recognize a shared antigen of AML: Molecular characterization of TCR genes

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Abstract

The identification of immunologically relevant tumor antigens is hampered by the difficulty of generating tumor-specific cytotoxic T cells (CTL). We present data demonstrating in vitro induction of autologous acute myelogenous leukemia (AML)-specific CTL. The specific T cell receptor has been identified and cloned. The CTL demonstrated specific lysis to autologous tumor blasts, but not to autologous BLCL or the NK-sensitive target K562. The clone secreted GM-CSF, TNFa, and IFNg when stimulated with AML blasts from 3 of 11 patients or cell lines tested, but not with K562 or autologous B-LCL. These three AML samples share a single HLA Class I antigen, HLA-A24. The T cell receptor genes identified by molecular methods are Vβ7.9-J2.3-Cβ2 and Vα17-J49-Cα.

Keywords: Acute leukemia; AML; T cell receptor; Leukemia antigen

1. Introduction

Acute myeloid leukemia (AML) is a hematological disorder marked by proliferation of myeloid progenitor cells. It is a heterogeneous disease [1] at the cellular and molecular level. Improvements in therapeutic regimens including chemotherapy, radiation, and marrow transplantation have resulted in improved survival, but the risk of relapse remains high. Long-term survival (greater than 5 years) is generally less than 50% [2,3]. A therapy capable of eradicating the residual tumor cells following conventional treatments could greatly improve survival [4].

The importance of the immune system, especially T lymphocytes in the control of AML, is demonstrated through the success of hematopoietic stem cell transplantation (HSCT). Recipients of allogeneic bone marrow transplantation have lower relapse rates than those who receive only chemotherapy or autologous transplantation [5]. T cell depletion of allogeneic marrow increases the risk of relapse [6]. The eradication of host tumor cells by donor-derived effector cells (graft versus leukemia) [7,8] and the use of donor lymphocyte infusions in the treatment of early AML relapse [9] support the role of T cell immunity in control of AML. Immunity to residual leukemia can develop even after autologous bone marrow transplantation [10]. The induction and expansion of the leukemia-specific effector cells could contribute to the elimination of residual disease without the complications of GVHD.

The graft versus leukemia phenomenon led to the recognition that minor histocompatibility antigens serve as tumor antigens and targets for cytotoxic T cells within the HSC graft. The existence of other leukemia specific antigens has been reported, and cytotoxic T cells (CTL) have been generated against peptides derived from these antigens including, WT1 [11] and proteinase 3 [12]. Takedatsu et al. [13] reports that CTL can be generated against epithelial antigens that are aberrantly expressed on hematologic malignancies. These CTL preferentially lyse leukemic cells that over-express the target antigens.

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Reports by Harrison et al. [14] and Panoskaltsis et al. [15] indicate that myeloid leukemic blasts cultured in the presence of cytokines that drive differentiation to a dendritic cell phenotype become potent antigen presenting cells. Charbonnier et al. [16] and Vereecque et al. [17] have observed that naïve autologous lymphocytes stimulated by leukemic APCs proliferate and yield CD8 cells that are specifically cytotoxic to leukemic blasts. Jahn et al. [18] describe a process to derive leukemia specific T cells from the bone marrow of untreated leukemic patients.

Clinical trials in melanoma have reported tumor regression in patients who received adoptive transfer of ex vivo expanded tumor infiltrating lymphocytes [19]. CTL effectors generated via culture with leukemic blasts-APCs offer a similar opportunity for treatment of myeloid leukemias. This study describes the induction and in vitro proliferation of a CD8+ clone that specifically recognizes a shared AML antigen in the context of HLA-A24. In addition, the T cell receptor genes used by the clone have been isolated. Induction and expansion of characterized cytotoxic T cells that recognize specific tumor could represent a viable treatment option for minimal residual disease. Identification of the T cell receptor genes also represents a potential for gene transfer immunotherapy.

2. Methods

2.1. Human subjects protection

This study was approved by the Research Subjects Review Board at the University of Rochester Medical Center. Informed consent was obtained from all participants. Research data were coded such that subjects could not be identified, directly or through linked identifiers, in compliance with the Department of Health and Human Services Regulations for the Protection of Human Subjects (45 CFR 46.10 {b} (4)).

2.2. Tumor cells

Leukemic blasts were obtained from an AML patient (AML-1) at the University of Rochester Medical Center and cryopreserved in vials at a concentration of 10^7 cells/ml. Following induction of remission, four serial blood draws were obtained. From these samples an EBV transformed B cell line (B-LCL) was established (as a negative control autologous line), immature dendritic cells were generated, and the remaining cells (predominantly T and B lymphocytes) were cryopreserved.

2.3. Generation of dendritic cells

Monocyte precursors were isolated from the peripheral blood mononuclear cells by either plastic adherence or selected via anti-CD14 coated magnetic beads. They were cultured in a medium containing human serum, penicillin, streptomycin, and the cytokines GM-CSF and IL-4 (1000 IU/ml of each) for one week. On day 7, leukemic blasts rendered apoptotic by exposure to 30 Gy irradiation were added to the culture. Cytokines IL-1β, IL-6, TNF-α and PGE-2 were added to induce maturation of the dendritic cells. At the end of culture surface expression of dendritic cell markers including CD83 and CD86 was assessed by flow cytometry.

2.4. Stimulation of T cells and limiting dilution

A CD8+ cytotoxic T cell population was isolated from the cryopreserved PBL by serial co-culture with apoptotic-blast pulsed autologous dendritic cells. Bulk lymphocytes underwent three rounds of stimulation. Prior to the third round of stimulation, the CD8+ cells were isolated using immunomagnetic beads coated with anti-CD8 antibody (MACS, Miltenyi Biotek). Following the third round of stimulation the T cells were tested for specific cytotoxicity against leukemic blasts. Limiting dilution assay isolated individual, reactive CD8+ clones. Cells were cultured in 96-well plates at concentrations of 1 or 10 cells per well in culture medium containing 50 U/ml rhIL-2, 10^3 irradiated tumor blasts, and 10^4 irradiated allogeneic feeder cells composed of PBMC from three healthy individuals. Media and feeder were replaced weekly. Cells in wells where clusters were observed were transferred and tested for cytokine production against autologous tumor, autologous B-LCL cells (negative control), and K562 (NK sensitive target).

2.5. Functional assays for CTL activity

2.5.1. Chromium release assay

Target cells (tumor blasts, B-LCL, K562) were incubated with radioactive ^{51}Cr (1 μCi/10^6 cells) for 1 h at 37 °C for 60 min. The targets were washed twice, resuspended at 10^5 cells/ml, and plated in 96-well plates 100 μl/well. T cell effectors were washed and resuspended at concentrations that would allow for a titrated response of effector:target (E:T) ratios; typical response curves ranged from 40:1 to 5:1.

2.5.2. Cytokine detection by ELISA

Stimulator cells (same as target cells in chromium assay) and T cells were co-cultured in 96-well plates for a period of 18–24 h at a ratio of 1:1 (2×10^4 each). At the end of culture, the supernatants were harvested following centrifugation. The presence of cytokine in the culture supernatants was assayed by ELISA (R&D systems). Cytokines assayed include IFN-γ GM-CSF, and TNF-α.

2.6. Rapid expansion protocol

T cell clones that were specific for tumor were expanded using a modified protocol developed by Dudley et al. [20]. Cultures of 10^3 T cells/well were cultured with 3×10^5 allogeneic filler/well (pools of three to five healthy individuals)

and OKT3 (30 ng/ml) in 96-well plates. IL-2 (300 IU/ml) and IL-7 (10 ng/ml) were added on day 2 of culture and supplemented every third day for at least 14 days.

2.7. HLA typing

HLA typing was performed using standard molecular methods. DNA was extracted from the PBMC fraction, and the presence of specific HLA Class I antigens/alleles identified by sequence specific primers (Pel Freez, Inc.).

2.8. T cell receptor variable chain cloning

The TCR α and β chains used by T cell clone 5 were identified using the method described by Moonka and Loh [21]. Reverse transcriptase (RT-PCR) was performed to prepare cDNA from mRNA extracted from clone 5. The degenerate, consensus primer described by Moonka and Loh binds a conserved region of both TCR variable regions; initial PCR was carried out under low stringency annealing. The 5′ end of the primer consisted of a non-degenerate anchor that lacks homology to the TCR; this primer was used in subsequent rounds under standard annealing conditions. Two different constant region primers were used in a nested fashion to increase specificity. The primers used were as described in the study by Moonka and Loh.

2.9. PCR conditions

5′ primer = anchor + consensus region; 3′ primer = CB1	
Cycle 1	94 °C, 4 min; 37 °C, 10 min; 72 °C for 30 s
Cycle 2–5	94 °C, 30 s; 37 °C, 10 min; 72 °C for 30 s
Pause PCR; add anchor primer at 1 uM	
Cycle 6–35	94 °C, 30 s; 58 °C, 30 s; 72 °C for 30 s
Round 2 PCR conditions; 1:50 dilution of round 1 product use 1 μl	
5′ primer = anchor; 3′ primer = CB2	
Cycle 1	94 °C, 4 min; 58 °C, 30 s; 72 °C for 30 s
Cycle 2–29	94 °C, 30 s; 58 °C, 30 s; 72 °C for 30 s

PCR control reactions included a no reverse transcriptase and a no template tube from the first and second rounds. Products were visualized under ethidium bromide staining in gel electrophoresis.

2.10. TOPO TA Cloning®

PCR products of the appropriate size (350 base pair) were cloned into chemically competent cells for sequencing. The TOPO TA cloning™ kit (Invitrogen) exploits the 3′ deoxyadenosine added to PCR products by *Taq* polymerase which permits the product to be ligated into a plasmid vector that contains 3′ thymidine overhangs. The vector was amplified by transfection into chemically competent *E. coli* cells selected on ampicillin containing agar plates. Bacterial colonies were picked; plasmid DNA was isolated using Perfectprep® Plasmid Mini kits (Eppendorf), and inserts removed by Eco R1 digestion. Resulting products were visualized by ethidium bromide gel electrophoresis. The expected product size based on primer positioning is approximately 330 bp. Samples that exhibited appropriate size were sequenced.

Once the variable, joining, and constant genes were identified, full-length TCR was cloned by designing primers for the specific 5′ and 3′ sequences. The sequencing was performed in two steps. Gel-purified products of the two reactions were subjected to a second fusion extension reaction. The products of the fusion extension were subjected to TOPO TA cloning as above.

3. Results

3.1. AML-1 T cells demonstrate specific lysis for autologous tumor blasts

Autologous DC for T cell stimulation were derived from PBMC as described in Section 2. Mature DCs were readily identified by a distinctive morphology characterized by cytoplasmic veils visualized under phase microscopy. They were further characterized by the presence of specific cell markers including CD83 and CD86 (B7) as detected by flow cytometry (Fig. 1). After three rounds of stimulation against autologous dendritic cells pulsed with irradiated tumor cells, the CD8+ population was tested for specific lysis (Fig. 2). While tumor specific cytotoxicity was observed, the activity was very low and the differential between tumor and normal was minimal (10% and 5%, respectively). This phenomenon had been observed in previous experiments (data not shown) and is similar to that reported by Spisek et al. [22] under similar conditions of stimulation. Since this was a bulk cultured cell line, the frequency of tumor specific T cells in the population was unknown. Limiting dilution culture was performed to isolate tumor specific clonal populations. A total of ten 96-well plates were prepared from which 22 wells grew during a 4-week period. Cells from these 22 wells were harvested and tested for tumor specific activity (Fig. 3). Five T cell clones produced IFN-γ when stimulated with autologous AML blasts as compared to control wells (autologous B-LCL and T cells with no stimulators). One of the five clones (clone 5) grew well and provided sufficient cells for further testing.

3.2. Clone from AML-1 recognizes a shared leukemia antigen

This T cell clone clearly recognizes the primary tumor blasts of the patient. To determine if this clonal T cell

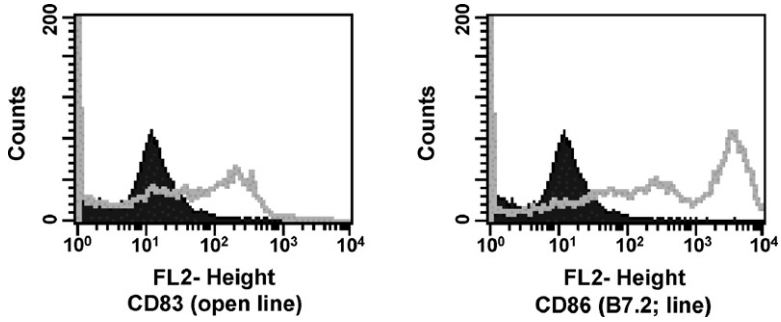

Fig. 1. Flow cytometry of mature human dendritic cells.

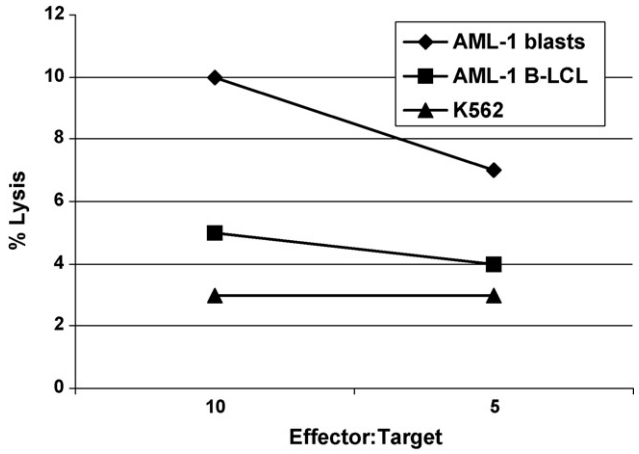

Fig. 2. Chromium release assay of bulk CD8+ T cell line from AML-1. Targets include autologous tumor blasts, autologous EBV-B cells, and K562 cells that are NK sensitive targets that lack MHC expression.

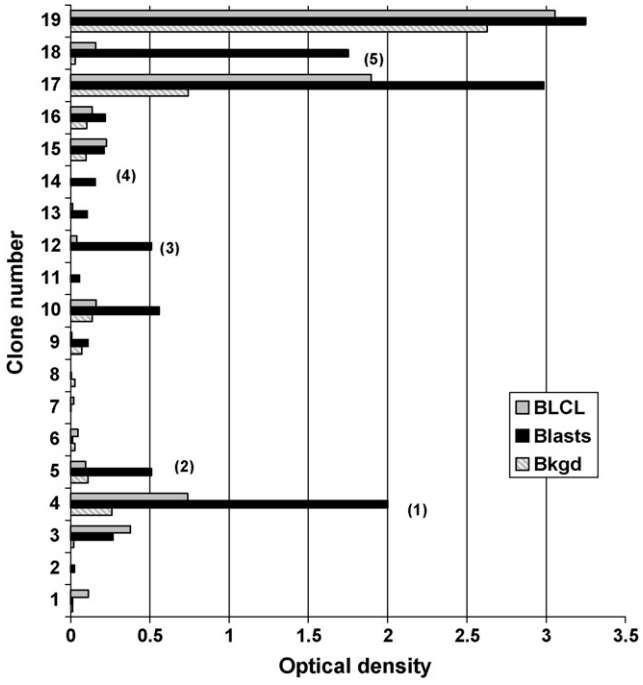

Fig. 3. IFN-γ ELISA of supernatants from clonal populations alone or stimulated with either autologous EBV-B cells or tumor blasts. Cells from five populations were selected for expansion and further testing. Only cells from clone 5 grew well.

population might also lyse blasts of other leukemias, a panel of HLA typed, leukemic blasts (Table 1) from nine AML patients and two AML cell lines (THP-1 and KG-1 obtained from the ATCC) were used as stimulators in the cytokine assay. Interestingly, this T cell clone produced high levels of both GM-CSF and IFN-γ (Fig. 4a) when cultured with the autologous blasts (AML-1), another primary tumor (AML-2) and cell line THP-1. Importantly, the T cells also effectively lysed autologous AML-1 blasts, AML-2 blasts and THP-1 targets, but not the autologous B-LCL lines or K562 when tested in a chromium release assay (Fig. 4b).

Table 1
Characterization and HLA type of AML blasts

Designation	Disease/FAB type	HLA type
AML-1	AML M5	A3, 24; B7, 15; Cw3, 7
AML-2	AML M5	A2, 24; B18, 72; Cw12
AML-3	AML M2	A11, 33; B35, 38; Cw3, 12
AML-4	AML unknown	A1, 11; B8, 44; Cw5, 7
AML-5	AML unknown	A1, 11; B44, 51; Cw5, 15
AML-6	AML unknown	A26, 31; B7, 55; Cw3, 7
AML-7	MDS/AML	A2; B35, 45; Cw6, 9
AML-8	AML M4	A26, 28; B14, 35; Cw4, 8
AML-9	AML M1	A2, 23; B44; Cw5, 16
THP-1	AML M5	A2, 24; B5
KG-1	AML	A30, 31; B35; Cw4

AML 1, AML2, and THP-1 share a single HLA Class I molecule, HLA-A24. None of the other eight leukemic blasts tested express this antigen or stimulate clone 5 T cells. In a separate experiment, clone 5 cells were tested against two melanomas and a prostate cell line all of which express HLA-A24, but they did not produce IFN-γ after 20 h incubation (data not shown). Thus T cell clone 5 recognizes a shared leukemia antigen in the context of HLA-A24.

3.3. T cell receptor

To demonstrate that clone 5 was, in fact, clonal we undertook to identify the specific TCR genes used. Molecular cloning was accomplished using a consensus primer specific

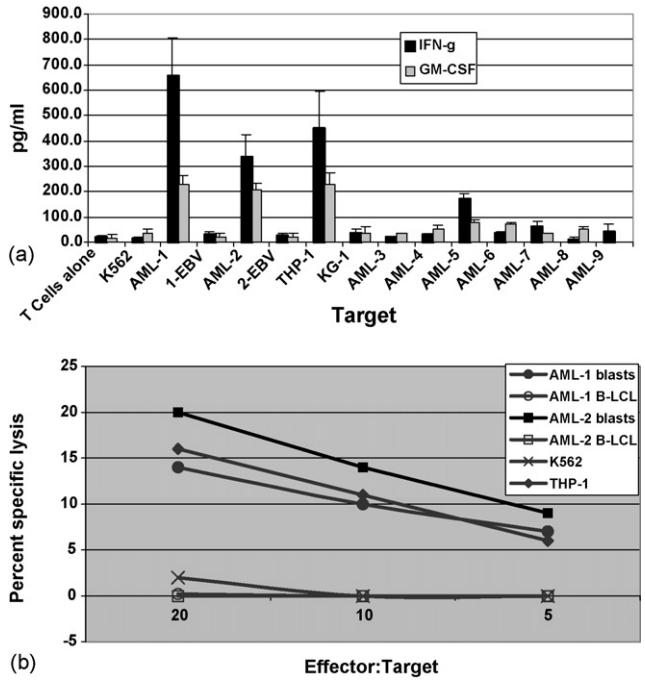

Fig. 4. (a) Clone 5 T cells generated from patient AML-1 produce GM-CSF and IFN-γ when cultured with autologous leukemic blasts (AML-1), tumor cells from an allogeneic patient (AML-2), and AML cell line THP-1 in the cytokine assay. AML1, AML2 and THP-1 express HLA-A24. (b) Clone 5 T cells lyse autologous tumor blasts, tumor from AML-2, and THP-1 but not the correlate B-LCL lines or K562.

for the second framework region of the variable chain of both the α and β genes [21]. The amplified product includes CDR 2, CDR3, the N-region, J-genes and the 5′ end of the constant chain. Nucleotide sequences of these products were blasted against the Immunogenetics (IMGT) Database [23]. Full-length products were amplified using the 3′ and 5′ sequences obtained from IMGT. Molecular cloning of cDNA from T cell clone 5 revealed that it uses Vα 17.1-Jα49.1-$C\alpha_1$ and Vβ 7.9-Jβ2.3-Cβ2. The protein sequence is detailed below.

Vα 17.1

METLLGVSLVILWLQLARVNSQQGEEDPQALSIQ-EGENATMNCSYKTSINNLQWYRQNSGRGLVHLILIR-SNEREKHSGRLRVTLDTSKKSSSLLITASRAADTASY-CATDNTGNQFYFGTGTSLTVIPNIQNPDPAVYQLRDS-KSSDKSVCLFTDFDSQTNVSQSKDSDVYITDKTVLD-MRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTF-FPSPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILLL-KVAGFNLLMTLRLWSS.

Vβ 7.9

MGTSLLCWMALCLLGADHADTGVSQNPRHKITK-RGQNVTFRCDPISEHNRLYWYRQTLGQGPEFLTYFQ-NEAQLEKSRLLSDRFSAERPKGSFSTLEIQRTEQGDS-AMYLCASSPPWTDTQYFGPGTRLTVLEDLKNVFPPE-VAVFEPSEAEISHTQKATLVCLATGFYPDHVELSWW-VNGKEVHSGVSTDPQPLKEQPALNDSRYCLSSRLRV-SATFWQNPRNHFRCQVQFYGLSENDEWTQDRAKP-VTQIVSAEAWGRADCGFTSESYQQGVLSATILYEILL-GKATLYAVLVSALVLMAMVKRKDSRG.

4. Discussion

The T cell repertoire is primarily established in the thymus [24]. T cells that demonstrate high affinity for self-peptides are actively deleted (negative selection) and those with low or absent recognition for self die of neglect (lack of positive selection). Cells that interact moderately with MHC-peptide are positively selected. Therefore, the final repertoire consists of T cells that have intermediate affinity for self MHC [25]. This allows some auto-reactive T cells to exist in the peripheral T cell repertoire. Potential autoimmunity is controlled via peripheral tolerance mechanisms including T cell apoptosis, anergy, and the influence of T regulatory cells [26].

Naïve T cells become activated when they encounter cognate MHC-peptide on the surface of APCs. The sensitivity of the T cell to a specific antigen is determined both by the binding strength of peptide for MHC and the affinity of the TCR for the MHC peptide complex [27]. Co-stimulation in the form of adhesion molecules and accessory molecules that signal cytokine production are also required for activation of T cells [28]. Once activated, CTL proliferate and kill cells that express their specific MHC-peptide complex, even when the antigen is present at reduced concentrations.

The challenge for tumor immunology is to isolate potentially autoreactive T cells that escape thymic selection and activate them to recognize antigens that are aberrantly expressed or over-expressed on tumor cells. The data presented here successfully address the challenge of isolating such T cells specific for AML. Lee et al. [29] and Azuma et al. [30] have generated T cells specific for tumor antigens by inoculation or co-incubation with peptides derived from tumor antigen. While CTL generated by such methods recognize cognate antigen, their functional avidity is often low, and clinically observable responses are infrequent. In an attempt to improve the avidity of the CTL, heteroclitic peptides containing amino acid substitutions intended to improve binding to MHC have been used [29]; however, improvement in clinical responses was not observed. Immunization with peptide may not produce optimally functional T cell responses. Antigen presented without appropriate co-stimulation or at a high dose may induce tolerance rather than activation.

The T cell repertoire produced by peptide immunization has not been well characterized. Stuge et al. [31] studied endogenous T cells from melanoma patients and T cells obtained after immunizing patients with melanoma antigenic peptides derived from MART-1 and gp100. T cells that bound MHC tetramers loaded with appropriate peptide were isolated. Individual T cell clones were expanded and analyzed for anti-tumor activity and TCR Vβ usage. They observed that in the immunized group, T cell responses to tumor cells were variable and exhibited a diverse use of Vβ genes. In contrast, T cells obtained endogenously (no vaccination) from two patients were tumor specific and exhibited

robust cytotoxicity directly ex vivo. They also demonstrated limited Vβ use; the dominant gene for both patients was Vβ17. While the peptide-immunized group also produced T cell clones that used the Vβ17 gene, a large number of other peptide-specific clones that did not recognize tumor were also produced. The potential efficiency of the Vβ17 clone was diluted by the expansion of many other antigen specific, but functionally ineffective clones in the immunized population.

The approach we used was to isolate endogenous, functionally active, tumor-specific T cells. The T cells demonstrate specificity against an antigen that is naturally presented by the tumor, and not a synthetic peptide. The T cell clone exhibits specific lysis of AML blasts and is HLA-A24 restricted. The peptide antigen is currently unknown.

A therapeutic strategy that has proved effective in treating melanoma is the adoptive transfer of tumor reactive T cells [19]. Tumor infiltrating lymphocytes (TIL) are isolated, expanded ex vivo with anti-CD3 and IL-2 and then transferred back to the patient after chemotherapy and lymphodepletion. Rosenberg and Dudley [19] reported that 18/35 patients had objective clinical response and 4 patients had complete responses using this approach. The advantage here is that large numbers of activated T cells selected for optimal lysis can by administered. A later report by Robbins et al. [32] noted significant correlation between tumor regression and the degree of persistence of adoptively transferred T cell clones. In leukemia, circulating CD8 cells represent TIL-equivalents and were easily isolated. As demonstrated in this study, activating the T cells was achieved in the presence of autologous blasts and dendritic cells generated from monocytes isolated from peripheral blood.

One prerequisite to adoptive therapy is the production of sufficient quantities of tumor-specific T cells. Non-transformed T cells do not expand indefinitely even in the presence of growth factors such as IL-2. In addition, the adoptively transferred T cells may be subject to negative feedback or peripheral tolerance mechanisms. Some tumors express FasL (CD95L) the ligand for the pro-apoptotic receptor Fas (CD95) present on T cells [33]. AML blasts have been shown to release immunosuppressive factors such as TGF-β [34], soluble CD25 (IL-2 receptor) [35], and soluble adhesion molecules [36] that might interfere with an adoptive immunotherapy. In fact, we observed that the cytotoxic response noted early in the culture of the initial T cell line, declined upon continued culture in the presence of tumor blasts without dendritic cells.

A solution to mitigate these limitations is to isolate and transfer the TCR antigen-recognition unit. T cell receptor transfer has proved to be effective. Rubinstein et al. [37] demonstrated that transfer of TCR genes from OT-1 (ovalbumin) specific TCR transgenic mice into mature T cells resulted in effector cells of avidity and lytic ability similar to the parental T cells. Schaft et al. [38] used two peptide specific TCR constructs to show that TCR transfer did not alter peptide fine specificity. Clay et al. [39] has demonstrated that the transfer of melanoma antigen MART-1 specific TCR to peripheral blood lymphocytes confers tumor specific reactivity. This strategy is currently being tested in clinical trials.

Hematologic malignancies are excellent candidates for T cell immunotherapy. While some cancers have been shown to down-regulate or turn off MHC expression [40], many leukemias express Class I molecules [41] that are the target of CD8+ cytotoxic T cells. The three malignancies recognized by clone 5 are AML M5, monocytic leukemias. This subset generally also expresses higher levels of co-stimulatory molecules such as CD40 and CD86, and adhesion molecules such as CD11a [42], which may make them even better targets for T cell immunotherapy, especially if the expression of these molecules can be up-regulated by concomitant cytokine therapy.

The restriction element for the TCR in this study is HLA-A24, a fairly common Class I antigen with a gene frequency from about 3% among African–Americans to more than 20% in North American Natives and Asians [43]. The T cells recognize both dominant alleles of the antigen HLA*A2402 and A*2403. A number of tumor-related antigens previously identified are presented by HLA-A24 including epitopes for SART-1 [44], cyclophilin B [45], and MAGE-1 [46]. While at this time the specific antigen recognized by the T cell clone 5 has not been characterized, the recognition element has been isolated. The isolated TCR will be useful in identifying this shared antigen of AML.

The approach described here successfully generated a tumor-specific T cell clone that recognizes a shared antigen presented by HLA-A24 in AML. The T cell receptor used by this cell has been cloned. The T cell clone and its TCR genes are valuable tools that may be used in the identification of the antigen and independently for immunotherapy.

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Contributions. Todd Belanger performed some of the technical assays; he is currently affiliated with Wyeth, Inc. Maurice Zauderer was thesis advisor to the corresponding author. Deepak Sahasrahbudhe was a member of the advising committee and clinician consultant for the project.

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