

Targeting ALDH^{bright} Human Carcinoma-Initiating Cells with ALDH1A1-Specific CD8⁺ T Cells

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Abstract

Purpose: Cancer-initiating cells (CIC) are considered to represent the subpopulation of tumor cells that is resistant to conventional cancer treatments, highly tumorigenic in immunodeficient mice, and responsible for tumor recurrence and metastasis. Based on an elevated aldehyde dehydrogenase (ALDH) activity attributable to ALDH1/3 isoforms, ALDH^{bright} cells have been identified and isolated from tumors and shown to have characteristics of CIC. The ALDH1A1 isoform was previously identified as a tumor antigen recognized by CD8⁺ T cells. This study examines the ability of ALDH1A1-specific CD8⁺ T cells to eliminate ALDH^{bright} cells and control tumor growth and metastases.

Experimental Design: ALDH^{bright} cells were isolated by flow cytometry using ALDEFLUOR from HLA-A2⁺ human head and neck, breast, and pancreas carcinoma cell lines and tested for their tumorigenicity in immunodeficient mice. ALDH1A1-specific CD8⁺ T cells were generated *in vitro* and tested for their ability to eliminate CICs *in vitro* and *in vivo* by adoptive transfer to immunodeficient mice bearing human tumor xenografts.

Results: ALDH^{bright} cells isolated by flow cytometry from HLA-A2⁺ breast, head and neck, and pancreas carcinoma cell lines at low numbers (500 cells) were tumorigenic in immunodeficient mice. ALDH^{bright} cells present in these cell lines, xenografts, or surgically removed lesions were recognized by ALDH1A1-specific CD8⁺ T cells *in vitro*. Adoptive therapy with ALDH1A1-specific CD8⁺ T cells eliminated ALDH^{bright} cells, inhibited tumor growth and metastases, or prolonged survival of xenograft-bearing immunodeficient mice.

Conclusions: The results of this translational study strongly support the potential of ALDH1A1-based immunotherapy to selectively target CICs in human cancer. *Clin Cancer Res*; 17(19); 6174–84. ©2011 AACR.

Introduction

Cancer-initiating cells (CIC) are characterized as a subpopulation of tumor cells in tumors which exhibit "stem cell-like" properties such as self-renewal, chemo- and radioresistance, and high tumorigenicity at low cell num-

bers in immunodeficient mice (1–5). Therefore, they are considered responsible for tumor recurrence and metastasis. In several types of tumors, cell populations enriched for cancer-initiating activity are being readily identified and isolated by flow cytometric analysis using the ALDEFLUOR reagent on the basis of their high level of aldehyde dehydrogenase (ALDH) activity and can be referred to as ALDH^{bright} cells (6–12). The ALDH activity detected by this reagent is primarily attributed to members of the ALDH1 and ALDH3 family of ALDH isoforms. The ability to readily identify and isolate ALDH^{bright} cells by flow cytometry is facilitating the efforts to develop therapeutic approaches that would target CICs and elicit long-term and effective responses in subjects with cancer (13). ALDH1-targeted immunotherapy represents such an approach, as in a previous study we have shown that the ALDH1A1 isoform can mediate the recognition and lysis of ALDH1A1⁺ squamous cell carcinoma of the head and neck (SCCHN) cell lines by cognate CD8⁺ CTLs. Relevant to the potential clinical use of ALDH1A1-specific CTL-based immunotherapy, ALDH1A1-specific CTLs recognize neither normal differentiated cells, such as fibroblasts, unless they are transfected to express high levels of ALDH1A1, nor

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Translational Relevance

Tumor cells expressing high levels of aldehyde dehydrogenase (ALDH) have been identified by flow cytometry as ALDH^{bright} cells and shown to have the properties attributed to cancer-initiating cells (CIC), which are resistant to conventional cancer treatments and considered responsible for recurrence and metastasis. Pertinent to developing immunotherapy for targeting CICs, these cells express ALDH1A1, a tumor-associated antigen recognized by HLA class I restricted, CD8⁺ T cells, which can be induced/generated *in vitro* and are present in human subjects with cancer.

This study shows that ALDH^{bright} cells are sensitive to cytolysis by ALDH1A1-specific CTLs *in vitro*. In preclinical models of human tumor xenografts growing in immunodeficient mice, adoptive therapy with ALDH1A1-specific CD8⁺ T cells was shown to target ALDH^{bright} cells and inhibit xenograft growth and metastases, or prolong survival. Our results show the usefulness of ALDH1A1 as a target of T cell-based immunotherapy to eliminate CICs in tumors.

normal CD34⁺ hematopoietic stem cells. The latter express ALDH1A1 at a level that is higher than that found in most normal differentiated cells and tissues but lower than that in CICs (14).

In the present study, we have investigated the ability of *in vitro* generated ALDH1A1-specific CTLs to eliminate ALDH^{bright} cells present in HLA-A2⁺ human carcinoma cell lines, xenografts, and surgically removed lesions *in vitro* and the antitumor activity of adoptive immunotherapy with ALDH1A1-specific CTLs *in vivo*. In addition, we have analyzed the expression of ALDH1A1 and HLA class I antigen (Ag) in normal liver, as normal hepatocytes have been reported to express a high level of this ALDH1 isoform (15) and therefore represent a potential concern in implementing ALDH1A1-based immunotherapy.

Materials and Methods

Human cell lines, tumor specimens, and blood

The human SCCHN cell lines used in these studies have been previously described (14). The MDA-MB-231 breast and MIA PaCa-2 pancreatic carcinoma cell lines were obtained from American Type Culture Collection. The luciferase-transfected MDA-MB-231-Luc cell line was obtained from Xenogen. KT-64 feeder cells were generously supplied by Dr. Bruce Levine (University of Pennsylvania, Philadelphia, PA; refs. 16–18). Cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, 2 mmol/L L-glutamine, 50 µg/mL streptomycin, and 50 IU/mL penicillin (Life Technologies, Inc.). Tumor and blood specimens were obtained from consented subjects with SCCHN and pancreatic cancer under the auspices of the University of Pittsburgh Tissue Bank with Institutional

Review Board (IRB) approval #991206 and Massachusetts General Hospital IRB approval #08-265, respectively. Blood was obtained from HLA-A2⁺ normal donors with IRB approval #980633.

Antibodies

ALDH1A1-specific rabbit monoclonal antibody (mAb; catalogue no. ab52492) was purchased from Abcam. The HLA-A, -B, -C, -E, -F, -G Ag-specific mAb W6/32 (IgG_{2a}), HLA-A2, -A28 Ag-specific mAb KS1 (IgG₁), HLA-DR Ag-specific mAb L243 (IgG_{2a}), HLA-A heavy chain-specific mAb HC-2A (IgG₁), and HLA-B, -C heavy chain-specific mAb HC-10 (IgG_{2a}) have been previously described (19–23). APC anti-CD5, FITC anti-CD8, ECD anti-CD45RA, and PC7 anti-CCR7 mAb were purchased from BD Biosciences. Rabbit anti-histone H3 phosphoserine10 mAb was purchased from Cell Signaling Technology, Inc. The Apop-Tag Plus Peroxidase In Situ Apoptosis Detection Kit was purchased from Millipore Corp.

Flow cytometric analysis of cell surface-stained cells

Tumor cell lines were harvested with 1 mmol/L EDTA (Sigma), and xenografts and lesions were disaggregated with Collagenase Type 4 (Worthington Biochemical). Duplicate aliquots of tumor cell samples were incubated with ALDEFLUOR (Stem Cell Technologies), with or without the ALDH inhibitor diethylaminobenzaldehyde (DEAB; control) according to the manufacturer's instructions (14). To identify ALDH⁺ and ALDH^{bright} cells, the control aliquot of the sample was analyzed by flow cytometry and set for detection of 0.2% or more ALDH⁺ and 0% ALDH^{bright} cells in the aliquot. Using this cutoff value, the test aliquot was analyzed to identify its ALDH⁺/ALDH^{bright} cell content. The results for human tumor cell lines or lesion samples can vary depending on *in vitro* propagation of cell lines, lesion disaggregation conditions, and/or reagent lot. Cells were sorted with a Dako-Cytomation MoFlo sorter (Dako North America) at 1.5×10^3 events/s.

Cells were surface stained for HLA class I Ag-specific mAb by standard procedures. Flow cytometry was carried out with an FC500 cytometer (Beckman Coulter), which was calibrated daily with fluorescent beads; all samples were run by identical settings to collect a minimum of 10,000 gated events, when possible. Analyses were conducted with EXPO32 ADC software (Beckman Coulter) or Summit V4.3 (Dako).

Real-time reverse transcriptase PCR analysis of ALDH1 mRNA

Expression of ALDH1 isoform mRNA relative to that of β -glucuronidase (an endogenous control or housekeeping gene) mRNA was determined with commercially available and custom-designed ALDH1 isoform primer and probe sets and the Applied Biosystems 7700 Sequence Detection Instrument as previously described (14). The following primers/probe sets were used to measure ALDH1A1 mRNA: forward 5'-cgcaagacaggcttttcag-3', reverse 5'-tgtataatagtcgcccctctc-3', probe 5'-FAM-attggatcccctggcgtactatggat-3';

and ALDH1A2 mRNA, forward 5'-agctttgtgctgtggcaata-3', reverse 5'-gatgagggctccatgtaga-3', probe 5'-FAM-taagccagcagagcaaacaccactcag-3'. Applied Biosystems TaqMan Gene Expression Assay systems Hs00167476_m1 and Mm03003537_s1 were used to measure ALDH1A3 mRNA and β -glucuronidase mRNA, respectively.

Tumorigenicity of ALDH^{bright} cells in immunodeficient mice

ALDH^{bright} and ALDH^{neg} cells sorted from tumor cell lines were collected in 2 mL RPMI-1640 medium with 20% FBS and irradiated (300 Gy) bulk parental tumor cells, centrifuged, and the supernatant saved for later use. The pellets were suspended in a predefined volume of the saved supernatant and equal volume of Matrigel (BD Biosciences) so that a 100 μ L aliquot contained 500 sorted ALDH^{bright} or ALDH^{neg} tumor cells and 1×10^4 irradiated carrier/feeder cells. These aliquots were injected subcutaneously in the right and left flanks or intraperitoneally, respectively, in groups of NOD.CB17-Prck^{scid}/J [NOD/severe combined immunodeficient mice (SCID); The Jackson Laboratories] female (6–8 weeks of age) mice each. The tumorigenicity of MDA-MB-231-Luc cells was monitored by bioluminescence imaging with a Xenogen IVIS 50 instrument (Xenogen) according to the manufacturer's recommended protocol at the University of Pittsburgh Cancer Institute In Vivo Imaging Facility.

HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells

HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells were induced/expanded by *in vitro* stimulation (IVS) of CD8⁺ T cells isolated from peripheral blood obtained from normal HLA-A2⁺ donors with either ALDH1A1₈₈₋₉₆ peptide-pulsed autologous dendritic cells and OKT-3 mAb-activated KT64 feeder cells (the ratio of CD8⁺ T cells:dendritic cells:KT64 cells being 2:1:2) or ALDH1A1₈₈₋₉₆ peptide-pulsed artificial antigen-presenting cells (16–18). The yields of effector cells using artificial antigen-presenting cells as stimulators was 3-fold greater than that using peptide-pulsed dendritic cells and feeder cells and more than 10-fold greater than the use of peptide-pulsed dendritic cells only (data not shown). CD8⁺ T cells obtained from HLA-A2⁺ normal donors and IVS with the HLA-A2-restricted, HIVgag₃₆₂₋₃₇₀ peptide were used as controls in adoptive therapy experiments. Peripheral blood of HLA-A2⁺ normal donors and patients with SCCHN as well as IVS cultures was analyzed for HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells by flow cytometry using phycoerythrin-conjugated HLA-A2/ALDH1A1₈₈₋₉₆ peptide tetramer complexes obtained from the NIH Tetramer Facility as previously described (24).

Enzyme-linked immunosorbent spot assays

Enzyme-linked immunosorbent spot (ELISPOT) INF γ assays were conducted as previously described (14), using the ELISPOT 4.14.3 analyzer (Zeiss). Values were considered significantly different from control values based on the

double permutation test. Assay performance and reproducibility were monitored with aliquots of cryopreserved peripheral blood mononuclear cells (PBMC) obtained from a single donor stimulated with phorbol-12-myristate-13-acetate (10 ng/mL) and ionomycin (250 ng/mL; Sigma). The coefficient of variation for the assay was 15% ($n = 50$). For mAb blocking experiments, target cells were preincubated with either the blocking mAb or an isotype-matched mAb (10 μ g/mL) for 30 minutes at room temperature.

Flow cytometry-based cell-mediated cytotoxicity assay

Tumor cell lines, disaggregated xenografts, and lesions (5×10^5 cells) and HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells [2.5:1 effector/target (E/T) cell ratio] were incubated for 4 hours at 37°C, centrifuged, trypsinized, washed, incubated with ALDEFUOR, and analyzed for ALDH⁺ and ALDH^{bright} cells by flow cytometry. For mAb blocking experiments, target cells were preincubated with mAb (10 μ g/mL) for 30 minutes at room temperature.

Adoptive therapy with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells

Three distinct types of adoptive therapy experiments involving xenograft-bearing immunodeficient C.B-17 scid female mice (Taconic Farms) and HLA-A2-restricted, ALDH1A1-specific CD8⁺ T cells were carried out in this study. In a fixed endpoint experiment, C.B-17 scid mice ($n = 15$) were challenged with surgically implanted 5-mm pieces of a serial passage PCI-13-derived xenograft. Seven days later, the mice were placed by a stratified randomization based on tumor burden into 3 groups of 5 mice each and adoptive therapy initiated biweekly by intravenous injection with HLA-A2-restricted, ALDH1A1-specific CD8⁺ T cells, irrelevant HIVgag₃₆₂₋₃₇₀-specific CD8⁺ T cells (2×10^6 /mouse) or left untreated. Tumor volumes (mm^3) were calculated by the following formula: $(a \times b^2)/2$, where b is the smaller of the 2 diameter measurements (25). The experiment was terminated approximately 21 days later, the mice were euthanized, and xenografts were removed for analyses.

In the second model, experimental lung metastases of MDA-MB-231 cells were established in C.B-17 scid mice ($n = 9$) following intravenous injection of 1×10^6 cells to each mouse. On day 3, mice were randomly divided into 3 groups of 3 mice each. Group 1 was injected intravenously with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells (2×10^6 /mouse) twice per week for 5 weeks and then once per week for 4 weeks; group 2 was injected intravenously with irrelevant HIVgag₃₆₂₋₃₇₀-specific CD8⁺ T cells (2×10^6 /mouse) following the same schedule; and group 3 received no CD8⁺ T cells. All 3 groups received PEG-IL2 (equivalent of 6.6×10^4 IU/mouse) by intraperitoneal injection twice on the day CD8⁺ T cells were administered (26). All the mice were euthanized on day 65 and their lungs were harvested and fixed in 10% formalin for further analysis.

The third model used was a postsurgery and metastases survival model with a survival endpoint. NODscid female mice ($n = 27$) were challenged in the mammary fat pad with 1×10^6 MDA-MB-231 cells, and 30 days later, when the tumors were, on average, 0.8 cm in diameter, the xenografts were surgically removed. The mice were randomized into 3 groups of 9 mice each, 2 of which received weekly intravenous injections of either HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells or irrelevant HIVgag₃₆₂₋₃₇₀ peptide-specific CD8⁺ T cells (2×10^6 /mouse). All 3 groups of mice received PEG-IL2 (equivalent of 6.6×10^4 IU/mouse) by intraperitoneal injection twice on the day CD8⁺ T cells were administered.

Immunohistochemistry analyses

A liver tissue microarray (catalogue no. BN03011; US Biomax) was stained and analyzed for HLA class I Ag and ALDH1A1 expression in normal liver hepatocytes. The HC-10 and HC-A2 mAbs were used to stain for HLA class I Ag, and ALDH1A1 was detected with the rabbit anti-ALDH1A1 mAb by standard procedures.

Tumor areas in experimentally induced pulmonary metastases were analyzed with 4- μ m thick formalin-fixed paraffin-embedded sections of lung tissues stained with 0.5% alcoholic solution of hematoxylin (Sigma-Aldrich, Inc.). Photographs were taken with a Nikon Eclipse E800 microscope, and the areas of tumor nodules in 5 randomly selected fields per section (magnification 200 \times) were measured and calculated by the SPOT Advanced Imaging software (Diagnostic Instruments, Inc.). For analysis of proliferative and apoptotic cells in untreated and treated xenografts, formalin-fixed paraffin-embedded sections of xenografts were stained for histone H3 phosphoserine¹⁰⁺ and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling⁺ (TUNEL⁺) cells and analyzed by standard procedures. A minimum of 5 sections from each xenograft was stained, and 5 microscopic fields per section were counted manually in a double-blinded fashion by board-certified pathologists.

Statistical methods

Two-tailed Student's *t* test was carried out to interpret the differences between experimental groups. Kaplan-Meier analysis was used to calculate significance of median survival in the adoptive therapy in the postsurgery and metastases xenograft experiment.

Results

ALDH^{bright} cells present in established human carcinoma cell lines

Using flow cytometry and the ALDEFLUOR reagent, established human tumor cell lines and digests of tumor xenografts or surgically removed lesions were analyzed for their ALDH⁺ cell content. Sorting by flow cytometry was used to isolate the ALDH^{bright} cell population from the cell lines. The ALDH^{bright} cells identified in these samples had an ALDH mean fluorescence intensity (MFI) twice that of

the bulk ALDH⁺ cell population. A representative flow cytometric analysis of the human SCCHN cell line PCI-13 was done to identify ALDH⁺ and ALDH^{bright} cells in the cell line and set the parameters for sorting ALDH^{neg}, ALDH⁺, and ALDH^{bright} cells, together with the reanalysis of the sorted populations for ALDH⁺ cells and ALDH MFI, is shown in Figure 1A. Sorted ALDH⁺ PCI-13 cells were found to contain only 67% ALDH⁺ cells, whereas the sorted ALDH^{bright} cell population had a purity of 94%. The sorted ALDH^{neg} cells contained less than 1% ALDH⁺ cells and no ALDH^{bright} cells.

A panel of established human breast, pancreatic, and SCCHN cell lines and digests of disaggregated surgically removed pancreatic and SCCHN lesions were then analyzed by flow cytometry to identify their ALDH⁺ and ALDH^{bright} cell contents. Representative analyses are shown in Figure 1B, and the results are listed in Supplementary Table S1. In summary, the data indicate that the percentages of ALDH⁺ and ALDH^{bright} cells varied with each sample regardless of its tumor type or source. High percentages of ALDH⁺ cells in a sample did not automatically correlate with high percentages of ALDH^{bright} cells, but, on average, the ALDH^{bright} cell content was about 10% of the ALDH⁺ cell content. The ALDH^{bright} cell content ranged from a low of 0.02% in the SCCHN PCI-30 cell line to a high of 4.6% in the MIA PaCa-2 pancreatic carcinoma cell line-derived xenograft. The percentages of ALDH^{bright} cells in the MDA-MB-231 and MIA PaCa-2-derived xenografts were higher than those in the parental cell lines, whereas the ALDH^{bright} cell content of the PCI-13-derived xenograft was lower than that of the parental cell line. Additional passages of the xenografts did not result in significant changes in their ALDH^{bright} cell content (data not shown).

Tumorigenicity of ALDH^{bright} cells sorted from human carcinoma cell lines

To confirm that the ALDH^{bright} cell population was highly tumorigenic, a critical characteristic of CICs, ALDH^{bright} cells sorted from the PCI-13, MIA PaCa-2, and MDA-MB-231-Luc cell lines were tested for their tumorigenicity by challenging groups of 3 or 5 immunodeficient mice each at a dosage of 500 cells. Xenografts were established in 3 of 3 mice challenged with ALDH^{bright} PCI-13 cells, 2 of 3 mice challenged with ALDH^{bright} MIA PaCa-2 cells, and 4 of 5 mice challenged with ALDH^{bright} MDA-MB-231-Luc cells within 6 months of challenge (Supplementary Fig. S1). The tumorigenicity of the ALDH^{bright} MDA-MB-231-Luc cells was monitored by bioluminescence imaging. None of the ALDH^{bright} cell-derived xenografts can be attributed to the irradiated tumor feeder cells used in the inoculums, as ALDH^{neg} challenges, which also included the same number of irradiated tumor feeder cells, failed to yield xenografts in the same mice.

ALDH1A1 mRNA expression levels in ALDH^{bright} cells

According to its manufacturer, ALDH activity detected by the ALDEFLUOR reagent can be attributed to ALDH1 and ALDH 3 isoforms, with the emphasis on ALDH1 isoforms.

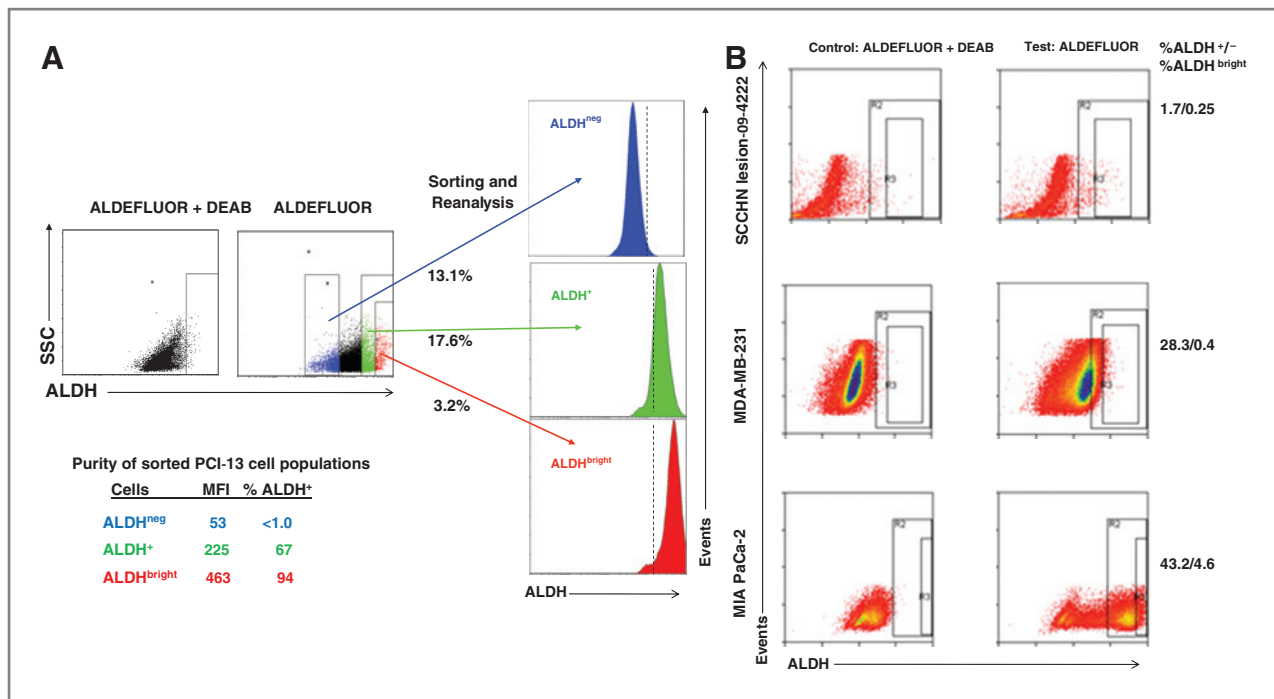


Figure 1. ALDH⁺ and ALDH^{bright} cells present in human carcinoma cell lines, cell line–derived xenografts, and a surgically removed SCCHN lesion. ALDH⁺ and ALDH^{bright} cells were identified by flow cytometric analysis following incubation of cells with ALDEFLUOR in the presence or absence of DEAB inhibitor. **A**, purity of sorted ALDH⁺ and ALDH^{bright} PCI-13 cells for ALDH⁺ cells: percentages of ALDH⁺ cells and ALDH MFI of each reanalyzed sorted population are indicated. The dotted lines in the reanalysis of the sorted populations indicate the gates set for identifying ALDH⁺ cells in the sample using ALDEFLUOR + DEAB. **B**, ALDH⁺ and ALDH^{bright} cells in the SCCHN PCI-13 cell line, a digest of a surgically removed SCCHN lesion, and MDA-MB-231 breast carcinoma and MIA PaCa-2 pancreatic carcinoma cell lines. The percentages of ALDH⁺ and ALDH^{bright} cells are listed.

Four ALDH1/3 isoforms have been identified, ALDH1A1, -1A2, -1A3, and ALDH3A1. A real-time reverse transcriptase PCR (qRT-PCR) analysis of the levels of expression of these 4 isoform mRNAs in bulk PCI-13 cells indicated predominant expression of ALDH1A1 mRNA compared with the other 3 isoform mRNAs. Little to no ALDH1A2 mRNA was expressed, and the level of ALDH1A1 mRNA was approximately 50 times greater than that of ALDH1A3 and ALDH3A1 mRNA, a finding consistent with ALDH1A1 contributing to the ALDH activity detected by ALDEFLUOR. Furthermore, the analysis indicated that ALDH^{bright} cells expressed approximately 8-fold higher level of ALDH1A1 RNA than bulk population of tumor cells (48.2 ± 5.6 vs. 6.2 ± 0.3). This result correlates well with the nearly 10 times higher ALDH MFI of ALDH^{bright} PCI-13 cells than that of the ALDH^{neg} PCI-13 cells. In addition, qRT-PCR analysis of the sorted ALDH^{bright} populations indicated that ALDH^{bright} PCI-13, MDA-MB-231, and MIA PaCa-2 cells uniformly express higher levels of ALDH1A1 mRNA than ALDH1A3 mRNA (Supplementary Table S2). Essentially no ALDH1A2 mRNA was detected in these cells (data not shown).

Detection of HLA-A2–restricted, ALDH1A1_{88–96} peptide–specific CD8⁺ T cells in the peripheral circulation of subjects with SCCHN

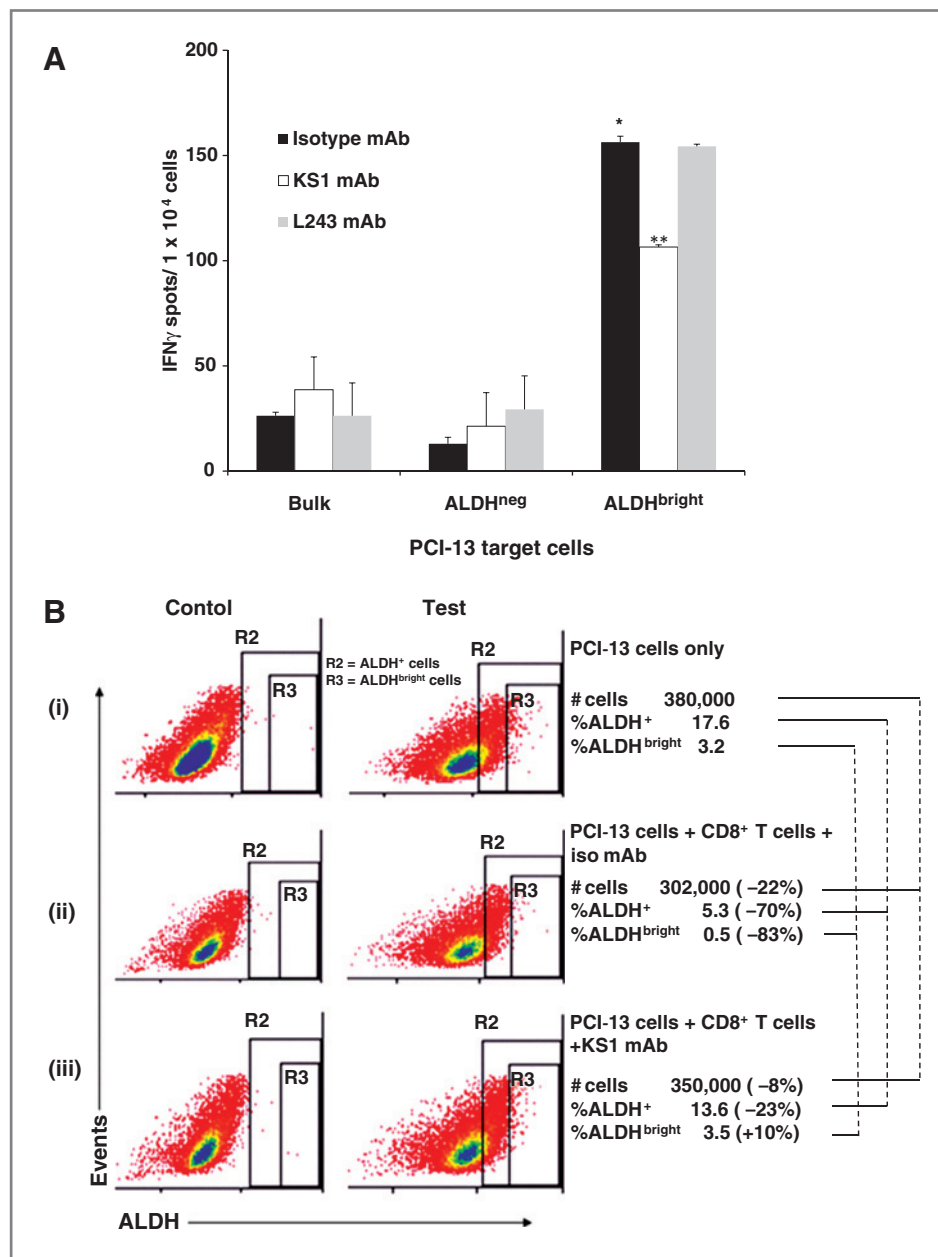
We previously identified ALDH1A1 as a tumor-associated antigen defined by HLA-A2–restricted, ALDH1A1_{88–96}

peptide–specific CD8⁺ T cells based on the ability of the ALDH1A1_{88–96} peptide to stimulate the *in vitro* induction/generation of these effector cells from PBMCs obtained from most normal donors, as well as a subject with SCCHN (14). *In vivo*, the immunogenicity of ALDH1A1_{88–96} peptide tetramer–based flow cytometric analysis of PBMCs obtained from HLA-A2⁺ subjects with SCCHN and normal donors. Representative results of this analysis are shown in Supplementary Figure S2. On the basis of a cutoff frequency of 1/8,000 determined with PBMCs obtained from HLA-A2^{neg} normal donors, the frequency of tetramer⁺ cells detected in PBMCs of HLA-A2⁺ normal donors was comparable with that of negative controls. In contrast, relatively high frequencies of tetramer⁺ cells in the range of 1/500 to 1/2,000 were detected in the peripheral circulation of subjects with SCCHN. The CCR7/CD45RA phenotypes of the tetramer⁺ CD8⁺ T cells varied with each subject; they had a predominately memory and terminally differentiated phenotype in subject 1, a predominately naive phenotype in subject 2, and a mixture of naive and terminally differentiated phenotypes in subject 3.

In vitro recognition of ALDH^{bright} cells by HLA-A2–restricted, ALDH1A1_{88–96} peptide–specific CD8⁺ T cells

The specificity of the HLA-A2–restricted, ALDH1A1–specific CD8⁺ T cells used in this study for the

Figure 2. *In vitro* recognition of ALDH^{bright} cells sorted from HLA-A2⁺ PCI-13 human carcinoma cell line by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells in ELISPOT IFN γ and flow cytometry-based CMC assays. **A**, recognition of sorted ALDH^{bright} PCI-13 cells but not sorted ALDH^{neg} or bulk PCI-13 cells by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells in ELISPOT IFN γ assays. The bulk populations of tumor cells used as targets were cells that had been incubated with ALDEFLUOR and collected without gating. Recognition of ALDH^{bright} cells blocked by HLA-A2, -A28-specific KS1 mAb but not affected by HLA-DR-specific L243 mAb. Assays conducted at an E/T ratio of 2:1. *, significant recognition ($P < 0.05$) of ALDH^{bright} cells relative to bulk or ALDH^{neg} cells. **, significant inhibition of recognition ($P < 0.05$) of ALDH^{bright} cells in the presence of KS1 mAb. **B**, flow cytometric analyses of ALDH⁺ and ALDH^{bright} cells present in SCCHN PCI-13 (5×10^5 cells) following incubation with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells at an E/T cell ratio of 2.5:1. Lysis was blocked by KS1 mAb. The decreases in the identified number of cells and percentages of ALDH⁺ and ALDH^{bright} PCI-13 cells incubated alone or with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells in the presence of isotype mAb or KS1 mAb are indicated.



ALDH1A1₈₈₋₉₆ peptide in ELISPOT IFN γ assays is shown in Supplementary Figure S3A. These effectors recognize ALDH^{bright} target cells but not the bulk cell population or ALDH^{neg} target cells sorted from the HLA-A2⁺ PCI-13 SCCHN cell line (Fig. 2A). Recognition of the ALDH^{bright} cells by the ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells was blocked by the HLA-A2-, HLA-28-specific mAb KS1 but was not affected by the HLA-DR-specific mAb L243. In an HLA-A2-restricted manner, these effector T cells also recognize ALDH^{bright} target cells, but neither bulk cell population nor ALDH^{neg} target cells sorted from the HLA-A2⁺ basal breast carcinoma MDA-MB-231 and pancreatic carcinoma MIA PaCa-2 cell lines (Supplementary Fig. S3B and C).

In vitro recognition by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells of ALDH⁺/ALDH^{bright} cells present in the established human carcinoma cell lines was also measured in a cell-mediated cytotoxicity (CMC) assay using flow cytometry. As indicated in Figure 2B, 83% and 70% decreases in the percentages of ALDH^{bright} and ALDH⁺ PCI-13 cells were observed following incubation of the tumor cells with the effectors, which can be attributed to the differential levels of ALDH1A1 expression in these cells. Recognition of ALDH^{bright}/ALDH⁺ cells was blocked by the HLA-A2, -28 Ag-specific mAb KS1. Importantly, comparable results were also obtained with cells derived from *in vivo* propagated tumor cells, namely, a PCI-13-derived xenograft and an HLA-A2⁺ surgically

removed SCCHN lesion (Table 1). These results further confirm that HLA-A2⁺ ALDH^{bright} tumor cells are recognized by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells.

Adoptive immunotherapy of tumor-bearing immunodeficient mice with ALDH1A1 peptide-specific CD8⁺ T cells

The efficacy of adoptive therapy with ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells was evaluated in immunodeficient mice bearing either subcutaneous xenografts derived from SCCHN PCI-13 cells or experimental or spontaneous pulmonary metastases derived from basal breast carcinoma MDA-MB-231 cells. In a fixed time point experiment involving immunodeficient mice bearing subcutaneous PCI-13-derived xenografts, adoptive therapy with intravenous ALDH1A1-specific CTLs was administered. The experiment was terminated on day 21 postimplantation to obtain sufficient residual xenograft specimens for subsequent analyses of their ALDH^{bright} cell content, as well as proliferation and apoptotic indices, measured by staining for histone H3 phosphoserine¹⁰⁺ cells (27, 28) and TUNEL⁺ cells, respectively. The results indicate that treatment of the xenografts with ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells, but not with irrelevant CD8⁺ T cells, significantly

inhibited their growth (Fig. 3A). This inhibition was concordant with a significant decrease in both their ALDH^{bright} cell content and proliferative index but a significant increase in the apoptotic index compared with xenografts obtained from the control groups of mice (Table 2 and Supplementary Fig. S4).

MDA-MB-231 cells readily form pulmonary metastases following intravenous injection or spontaneously following surgical removal of primary orthotopic xenografts. Adoptive therapy of mice bearing experimentally induced MDA-MB-231 pulmonary metastases by systemic administration of ALDH1A1-specific CD8⁺ T cells resulted in fewer and smaller tumor nodules with a significantly reduced total tumor area in the lungs of mice compared with that of the control groups of mice [irrelevant CTLs + interleukin (IL-2) and IL-2; Fig. 3B]. The total tumor area in the lungs of mice was quantified to determine the efficacy of the treatment in this experiment, because the metastatic lesions in the lungs of the control groups of mice had grown so extensively, had fused to form large tumor masses, and were not individually discernible (Fig. 3C; ref. 29).

In the clinically relevant, postsurgery and metastasis survival model, mice succumb primarily to lung metastases following surgical removal of the primary MDA-MB-231-derived orthotopic xenograft. Groups of immunodeficient

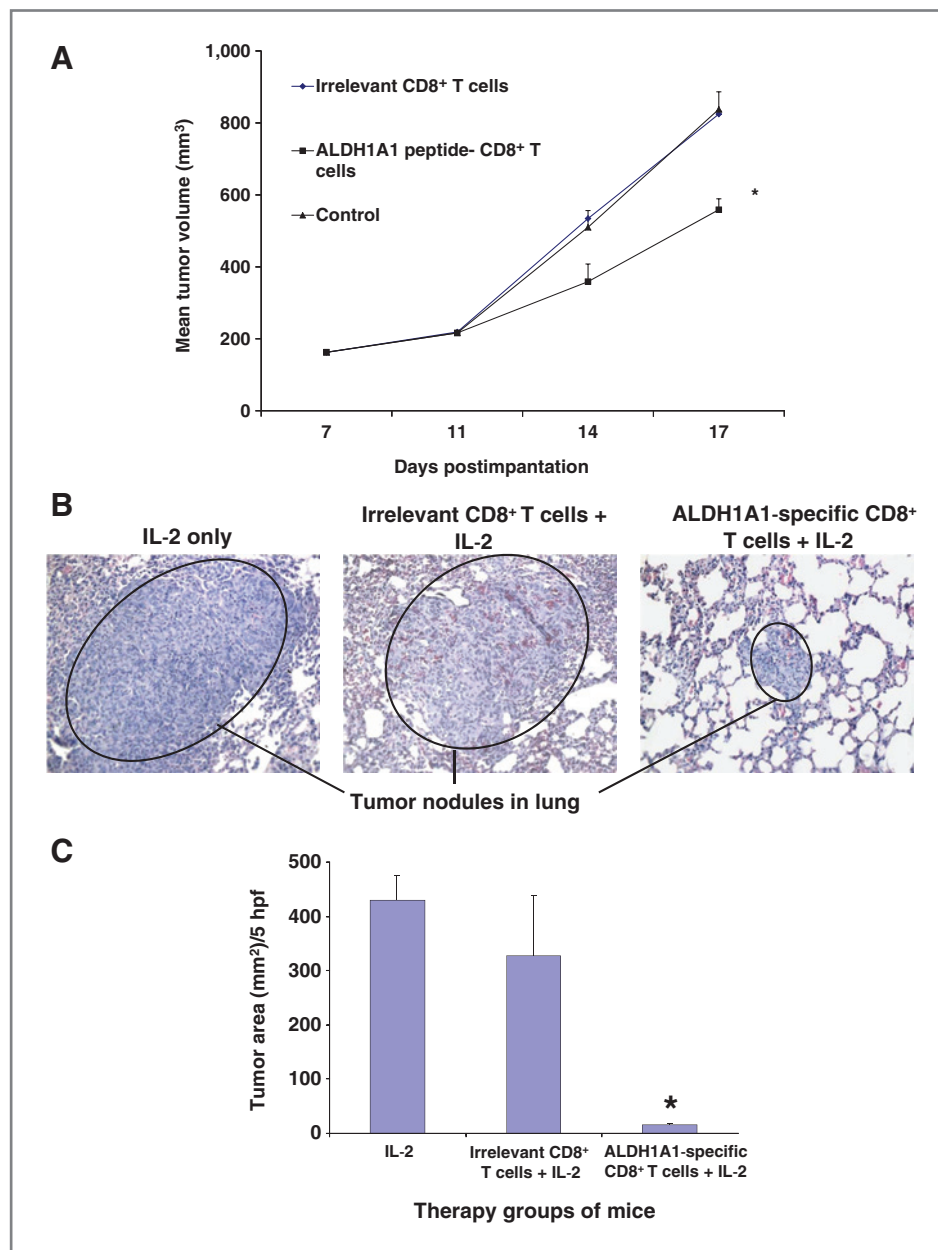
Table 1. *In vitro* recognition of ALDH⁺ and ALDH^{bright} cells present in human carcinoma cell lines, xenograft, and surgically removed lesion by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells in flow cytometry-based CMC assays

Cells	Cells only			Cells + ALDH1A1-specific CD8 ⁺ T cell + isotype mAb			Cells + ALDH1A1-specific CD8 ⁺ T cells + KS1 mAb		
	Cell number	% ALDH ⁺	% ALDH ^{bright}	Cell number	% ALDH ⁺	% ALDH ^{bright}	Cell number	% ALDH ⁺	% ALDH ^{bright}
PCI-13	380,000	17.6	3.2	302,000 (-22%)	5.3 (-70%)	0.5 (-83%)	350,000 (-8%)	13.6 (-23%)	3.5 (+10%)
MDA-MB-231	204,000	28.3	0.4	188,000 (-8%)	14.2 (-50%)	0.1 (-75%)	200,000 (-2%)	31.1 (+11%)	0.3 (-25%)
MIA PaCa-2	365,000	43.2	4.6	194,000 (-47%)	37.2 (-14%)	1.8 (-60%)	299,000 (-18%)	40 (-7%)	3.8 (-18%)
PCI-13 xenograft	350,000	13.1	2.1	213,000 (-29%)	3.5 (-73%)	0.6 (-72%)	298,000 (-15%)	13 (-5%)	1.7 (-22%)
SCCHN lesion 084124	150,000	3.4	0.9	100,000 (-33%)	0.9 (-74%)	0.1 (-89%)	ND ^a	ND	ND

NOTE: Flow cytometric analyses of ALDH⁺ and ALDH^{bright} cells present in SCCHN PCI-13, breast carcinoma MDA-MB-231, and pancreatic carcinoma MIA PaCa-2 cells and digests of a PCI-13-derived xenograft and a SCCHN lesion following incubation with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells at an E/T cell ratio of 2.5:1, followed by flow cytometric analysis with ALDEFUOR ± DEAB. A total of 5 × 10⁵ target cells were used with the exception of the analysis of the SCCHN lesion (2.5 × 10⁵ target cells). Lysis was blocked by HLA-A2, -A28-specific KS1 mAb. The percentages of ALDH⁺ and ALDH^{bright} cells in each sample following incubation with HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells in the presence of isotype mAb or KS1 mAb are indicated. The decreases in these values compared with the "cells-only" control are indicated in parentheses.

^aNot done due to insufficient cells.

Figure 3. Control of xenograft growth in immunodeficient mice by adoptive therapy with ALDH1A1-specific CD8⁺ T cells. Adoptive therapy of C.B-17 scid mice bearing xenografts derived from PCI-13 or MDA-MB-231 cells by intravenous injection of HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells. A, adoptive therapy of mice bearing 7-day subcutaneously growing PCI-13-derived xenografts by intravenous injection of ALDH1A1-specific CD8⁺ T cells. Mean tumor volume \pm SD (mm³) of PCI-13-derived xenografts in each group of mice of 5 mice each on the indicated days is shown. *, $P = 0.0001$ relative to untreated control. B and C, adoptive therapy of mice (3/group) bearing experimentally induced pulmonary metastases derived from the breast carcinoma MDA-MB-231 cell line. B, tumor lesions in representative formalin-fixed paraffin-embedded sections of lung tissue of indicated groups of treated mice bearing experimentally induced pulmonary metastases. Note differences in lesion sizes. C, total tumor area \pm SD (mm²) of lesions in lungs of the indicated groups of mice. *, $P < 0.001$ relative to mice treated with IL-2 only.



mice were treated with ALDH1A1-specific CD8⁺ T cells + IL-2, irrelevant CD8⁺ T cells + IL-2, or IL-2 only following their surgery. Only adoptive therapy with ALDH1A1-specific CTLs significantly prolonged their survival ($P < 0.001$) compared with the control groups of mice, as shown in Figure 4. All mice in the 2 control groups died from lung metastases by day 87 postsurgery, whereas 80% of the ALDH1A1-specific CD8⁺ T cells-treated mice exhibited no signs of disease at day 210 postsurgery. The results of these *in vivo* human tumor xenograft experiments show the efficacy of adoptive therapy with ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells to effectively target ALDH^{bright} cells and control tumor growth and metastases.

Immunohistochemical analysis of ALDH1A1 and HLA class I Ag expression in normal liver hepatocytes

In view of the potential clinical application of these results, we sought to address the question of whether ALDH1A1-based immunotherapy could target normal liver hepatocytes, which are reported to express a high level of ALDH1A1, and cause deleterious side effects (15). Therefore, we tested for the expression of HLA class I Ag and ALDH1A1 at the protein level in normal liver tissue by immunohistochemical staining with mAb. A liver tissue microarray composed of 63 cores derived from 3 nondiseased and 16 diseased livers (e.g., cirrhosis and fatty degeneration), 3 hepatocellular carcinomas, and an

Table 2. Effects of adoptive transfer of ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells on PCI-13-derived xenografts in immunodeficient mice

Group	ΔMTV ± SD ^a	% ALDH ^{bright} cells ^b	Proliferation index ^c	Apoptotic index ^d
Control	676 ± 45	1.3 ± 0.8	172 ± 18	22 ± 7
Irrelevant CD8 ⁺ T cells	662 ± 11	1.8 ± 0.5	176 ± 33	25 ± 7
ALDH1A1 ₈₈₋₉₆ peptide-specific CD8 ⁺ T cells	398 ± 33	0.4 ± 0.3	90 ± 25	41 ± 9
<i>P</i>	8 × 10 ⁻⁷	0.009	4 × 10 ⁻⁵	0.01

NOTE: See Material and Methods for the protocol used. Two-tailed Student's *t* test based on values of untreated control groups of mice was used to determine significance.

^aThe difference (Δ) in mean volume (mm³) of each tumor in a mice on days 7 and 20 was determined and the values expressed as mean tumor volume (MTV) ± SD for each group.

^bPercentages based on gated events.

^cMean ± SD of histone H3 phosphoserine10⁺ cells per tumor as analyzed by immunohistochemistry as detailed in Materials and Methods.

^dMean ± SD of TUNEL⁺ cells per tumor as analyzed by immunohistochemistry as detailed in Materials and Methods.

abnormal spleen was analyzed: 23 cores were considered to be normal liver tissue, 9 of which came from 3 non-diseased livers. Only 1 of these 9 cores stained for HLA class I Ag, and it showed weakly patchy staining. The remaining 14 "normal liver tissue" cores came from nondiseased regions of diseased livers; 2 showed strong cytoplasmic HLA class I Ag but weak ALDH1A1 expression, whereas 2 others showed HLA class I Ag membrane staining but no ALDH1A1 expression (see Supplementary

Table S3 and Fig. S5). In contrast, HLA class I Ag expression was prevalent in multiple cores of diseased tissue taken from diseased livers.

Discussion

The results of this translational preclinical study show that a subset of tumor cells in human carcinomas identified as ALDH^{bright} cells are recognized and eliminated *in vitro* and *in vivo* by HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells. In human tumor xenograft models, we have shown that adoptive transfer of ALDH1A1-specific CD8⁺ T cells inhibited growth of subcutaneously growing xenografts and experiment-induced lung metastases. In addition, following surgery to remove a primary tumor, this therapy inhibited spontaneous metastases and prolonged survival of mice.

It was recently reported that the ALDH activity in breast cancer stem cells detected by ALDEFLUOR is primarily due to ALDH1A3, rather than ALDH1A1, expression (30). In our study, however, we have shown that sorted ALDH^{bright} cells express higher levels of ALDH1A1 mRNA than ALDH1A3 mRNA. Nonetheless, as previously reported (14), the ALDH1A-specific CD8⁺ T cells used in this study recognize the ALDH1A1₈₈₋₉₆ peptide (LLYKLADLI) but not the highly related peptides derived from the ALDH1A2 (LLDKLADLV) and ALDH1A3 (LLHQLADLV) isoforms. Therefore, regardless of which ALDH1 isoform is prevalently expressed, the recognition of ALDH^{bright} cells by the ALDH1A₈₈₋₉₆ peptide-specific CD8⁺ T cells used in this study is independent of ALDH1A3 expression.

Although ALDH1A1 is expressed by many cell types, it is highly unlikely that ALDH1A1-based immunotherapy would induce toxicity. Normal stem cells such as hematopoietic stem cells, which express ALDH1A1 but at a lower level than detected in tumors, have been shown not to be recognized by ALDH1A1-specific CD8⁺ T cells (14).

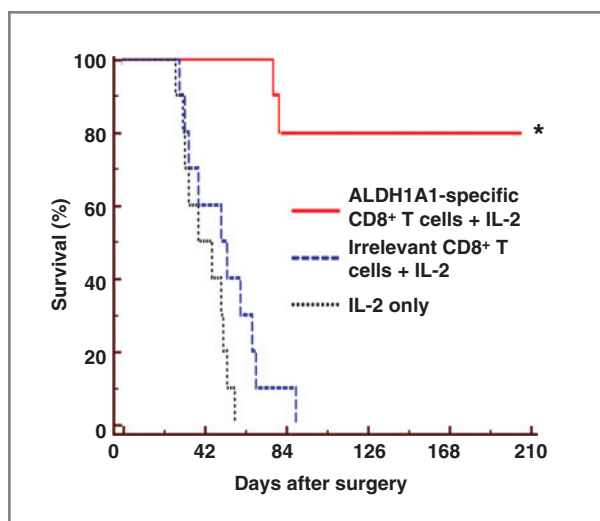


Figure 4. Adoptive therapy with ALDH1A1-specific CD8⁺ T cells of NODscid mice following surgical removal of primary MDA-MB-231 orthotopic xenograft. HLA-A2-restricted, ALDH1A1₈₈₋₉₆ peptide-specific CD8⁺ T cells were administered intravenously to groups of 9 mice each following surgical removal of their primary MDA-MB-231 orthotopic xenografts. Survival in each group of mice relative to time of surgery is shown. *, significant survival (*P* < 0.0001) of group of mice treated with ALDH1A1-specific CD8⁺ T cells and IL-2 relative to groups of mice treated with irrelevant CD8⁺ T cells and IL-2 or IL-2 only.

Furthermore, although ALDH1A1 is expressed by normal hepatocytes, in agreement with the information in the literature, we have shown that these cells express little to no HLA class I Ag on their cell surface; as a result, normal hepatocytes are highly unlikely to be recognized by HLA class I-restricted, ALDH1A1-specific CD8⁺ T cells (31–33).

Presently, there is little information about the recognition of CICs by HLA class I-restricted, CD8⁺ T cell effectors. To the best of our knowledge, only 3 studies have investigated this subject: 2 involve glioblastoma multiforme (GBM) stem cells isolated under selective culture conditions and the third involves sorted colon cancer stem cells identified as a side-staining population. Using a nontumor-related cytomegalovirus (CMV) antigen as a model tumor antigen (TA), Brown and colleagues (34) showed recognition of CMV-transfected GBM stem cells by CMV pp65 peptide-specific CTLs. Recognition of the targets, however, required targets pulsed with exogenous CMV pp65 peptide. This finding suggests that GBM stem cells expressed HLA class I Ag but required the exogenous peptide to form a sufficient level of HLA class I Ag-peptide complexes for recognition by the cognate CTLs. Di Tomaso and colleagues (35) detected defects in HLA class I Ag and APM component expression in the cultured population of GBM stem cells. As a result, recognition of these target cells by autologous antitumor CTLs required pretreatment with IFN γ to upregulate HLA class I Ag expression and, presumably, HLA class I Ag/TA peptide complexes, a common situation observed in targeting tumor cells with HLA class I-restricted, TA peptide-specific T cell effectors (36, 37). In the third study, Inoda and colleagues (38) showed that colon carcinoma stem cells are sensitive *in vitro* and *in vivo* to HLA class I-restricted CTLs recognizing an epitope derived from the tumor-associated centrosomal protein, 55-kDa protein CEP55, which is expressed by the tumor-initiating cells as well as the bulk population of cells in the colon carcinoma cell lines studied. Because ALDH1A1 is expressed by CICs present in colon carcinomas and gliomas (10, 39), targeting CIC populations in these tumors with ALDH1A1-specific CD8⁺ T cells is also possible and should be more selective.

Our results strongly support further development of strategies that would incorporate ALDH1A1-based immu-

notherapy to target CICs. The constraints of a practical evaluation of a T-cell–based immunotherapy using human xenograft mouse models required adoptive transfer of the immune effector cells. Using recombinant DNA or optimized traditional protocols, sufficient numbers of TA-specific T cells can be generated *in vitro* for adoptive T-cell–based immunotherapy; this strategy has been shown in recent years to yield beneficial clinical responses in subjects with cancer (40, 41). Nonetheless, the development and implantation of ALDH1A1-based immunotherapy need not preclude a vaccine-based approach.

In accordance with the cancer stem cell theory, the elimination of CICs should be the critical criteria used to define the efficacy of a therapy rather than only reduction in tumor volume. Our research shows for the first time the potential ability of an immunotherapy to achieve the objective of targeting CICs in tumors. Furthermore, because therapeutic protocols can promote tumor escape, our findings highlight the potential benefit that combining T-cell–based immunotherapy with other independent therapeutic modalities, such as TA-specific mAb and/or inhibitors of aberrantly regulated stem cell signaling pathways (13), could have on minimizing tumor escape.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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