Enhanced Function of Redirected Human T Cells Expressing Linker for Activation of T Cells That Is Resistant to Ubiquitylation

Naoki Kunii,^{1,*} Yangbing Zhao,^{1,2} Shuguang Jiang,¹ Xiaojun Liu,¹ John Scholler,¹ Lakshmi Balagopalan,³ Lawrence E. Samelson,³ Michael C. Milone,² and Carl H. June¹

Abstract

It is likely that the enhancement of signaling after antigenic stimulation, particularly in the tumor microenvironment, would improve the function of adoptively transferred T cells. Linker for activation of T cells (LAT) plays a central role in T cell activation. We hypothesized that the ubiquitylation-resistant form of LAT in cells would enhance T cell signaling and thus augment antitumor activity. To test this, human CD4⁺ or CD8⁺ T cells were electroporated with small interfering RNA (siRNA) to repress endogenous LAT and ubiquitylation-resistant LAT 2KR or wild-type LAT mRNA was introduced for reexpression. Significantly enhanced phosphorylation of LAT and phospholipase C- γ (PLC γ) was observed, and augmented calcium signaling after T cell receptor (TCR) triggering was observed in LAT 2KR-expressing T cells. TCR-induced calcium signaling was abrogated in LAT knockdown cells, but the baseline was higher than that of control siRNA-electroporated cells, suggesting a fundamental requirement of LAT to maintain calcium homeostasis. Redirected LAT 2KR T cells expressing a chimeric antigen receptor or an MHC class I-restricted TCR showed augmented function as assessed by enhanced cytokine secretion and cytotoxicity. These results indicate that interruption of LAT ubiquitylation is a promising strategy to augment effector T cell function for adoptive cell therapy.

Introduction

THE DEVELOPMENT OF redirected T cells encoding transgenic T cell receptors (TCRs) or chimeric antigen receptors (CARs) is a promising approach to overcome tolerance in patients with cancer and chronic infections (Greenberg and Riddell, 1999; Schumacher, 2002; Sadelain *et al.*, 2003; Johnson *et al.*, 2009; Bonini *et al.*, 2011; Kohn *et al.*, 2011). However, the clinical application of adoptive transfer has with some exceptions led to suboptimal efficacy for several reasons. To eliminate tumor, infused T cells must proliferate extensively *in vivo*, traffic to the tumor, and retain function in the immunosuppressive tumor microenvironment. Consequently, methods to enhance *in vivo* proliferation and function of transferred T cells may improve antitumor efficacy. In particular, decreasing the threshold for antigen signaling, increasing cytolytic activity, increasing resistance to tumor-

derived immunosuppressive mechanisms, and enforcing differentiation to helper T cell type 1 (Th1) or (Th17) lineages have promise (Paulos *et al.*, 2010). Approaches suggested to improve antigen signaling have included the incorporation of costimulatory molecules (Krause *et al.*, 1998; Kalos *et al.*, 2011), ablation of Cbl-b (Chiang *et al.*, 2007), and induction of diacylglycerol kinase- ζ deficiency (Riese *et al.*, 2011).

Linker for activation of T cells (LAT) is a type III transmembrane molecule that is critical for the coordination of signals triggered on T cell activation (Zhang *et al.*, 1998, 2000; Simeoni *et al.*, 2008). LAT contains a short extracellular domain, a single transmembrane-spanning region, and a long intracellular region containing nine tyrosines, of which five can be phosphorylated (Paz *et al.*, 2001; Zhu *et al.*, 2003). TCR signaling is initiated by phosphorylated Zap-70, which in turn phosphorylates LAT at conserved tyrosine residues (Horejsi *et al.*, 2004; Simeoni *et al.*, 2008). Phosphorylated

¹Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-5156.

²Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-4283.

³Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-4256.

*Present address: Head and Neck Surgery, Department of Otorhinolaryngology, Graduate School of Medicine, Chiba University, Chiba, Japan.

LAT recruits a number of signaling molecules resulting in calcium influx, nuclear factor of activated T cells (NFAT) activation, and ultimately expression of cytokine genes (Malissen *et al.*, 2005). Studies in mice with knockout or modified knock-in LAT molecules have revealed that LAT has a function in both the differentiation of T cells along the Th1 and Th2 pathways, as well as in the control of lymphocyte proliferation (Mingueneau *et al.*, 2009; Roncagalli *et al.*, 2010; Shen *et al.*, 2010). The function of LAT in post-thymic mature T cells is relatively unknown, and the function in human T cells is not well studied, as most studies to date have been done in mouse T cells. In one study we reported that expression of a ubiquitylation-resistant form of LAT in Jurkat cells was more stable than that of wild-type LAT and induced the enhancement of downstream signaling molecules and calcium flux (Balagopalan *et al.*, 2011). On the basis of these findings, we hypothesized that expression of ubiquitylation-resistant LAT in primary postthymic mature human T cells might enhance T cell signal transduction and function. We show that knockdown of wild-type LAT and expression of ubiquitylation-resistant LAT has the potential to generate engineered T cells with increased therapeutic efficacy.

Materials and Methods

Isolation, culture, and electroporation of primary human T cells

Primary human CD4⁺ T and CD8⁺ T cells were isolated from healthy volunteer donors after leukapheresis by negative selection, using RosetteSep kits (STEMCELL Technologies, Vancouver, BC, Canada). All specimens were collected under a university institutional review board-approved protocol and written informed consent was obtained from each donor. CD4⁺ and CD8⁺ T cells were expanded by CD3/CD28 bead stimulation (immunomagnetic beads to which CD3-specific and CD28-specific monoclonal antibodies had been conjugated; provided by the Clinical Cell and Vaccine Production Facility of the University of Pennsylvania, Philadelphia, PA) at a 1:1 cell-to-bead ratio in culture medium (RPMI 1640 [Invitrogen, Carlsbad, CA], 10% fetal calf serum [FCS; Gemini Bio-Products, West Sacramento, CA], penicillin [100 U/ml; Invitrogen], and streptomycin [100 µg/ml; Invitrogen]), with CD8⁺ T cells supplemented with human interleukin (IL)-2 (Chiron, Emeryville, CA) every other day to 300 IU/ml. After 10 days, these *in vitro*-expanded T cells were collected and cryopreserved. Cells were thawed and incubated for 24 hr before electroporation with *in vitro*-transcribed (IVT) RNAs, using a BTX CM830 system (Harvard Apparatus BTX, Holliston, MA) as previously described (Zhao *et al.*, 2010) and immediately placed into prewarmed culture medium.

Preparation of small interfering RNA and *in vitro* transcription of RNA

The small interfering RNA (siRNA) targeting human LAT at CCAACAGUGUGCGAGCUA (nucleotides 311 to 329) and the nontargeting control siRNA were purchased from Integrated DNA Technologies (Coralville, IA). Six constructs were cloned into the RNA transcription vector pGEM: (1) yellow fluorescent protein (YFP)–LAT wild-type (LAT WT)

fusion protein, (2) YFP–ubiquitylation-resistant LAT fusion protein, in which K52 and K204 were replaced with arginines (LAT 2KR) (Balagopalan *et al.*, 2011), (3) YFP–phosphorylation-resistant LAT fusion, in which Y132, Y171, Y191, and Y226 were replaced with phenylalanines (LAT 4YF) (Supplementary Fig. S1; supplementary data are available online at www.liebertonline.com/hum), (4 and 5) the α and β chains, respectively, of 1G4 HLA-A2-restricted NY-ESO-1 TCR (Zhao *et al.*, 2005), and (6) chimeric antigen receptor (CAR) of anti-CD19- ζ (Barrett *et al.*, 2011). In addition, the codons of LAT WT, LAT 2KR, and LAT 4YF were changed within the siRNA target (nucleotides 311 to 329), so that the amino acid sequence remained conserved but was resistant to siRNA degradation. IVT RNA was transcribed from *SpeI*-linearized plasmids, using a T7 mScript standard mRNA production system (CELLSCRIPT, Madison, WI) according to the manufacturer's protocol.

For knockdown of endogenous LAT, 1 nmol (12.7 µg) of LAT-targeting siRNA or control siRNA was used with or without various amounts of YFP–LAT fusions and coelectroporated into CD4⁺ T or CD8⁺ T cells (5×10^6 /100 µl). To analyze the cytotoxicity of these cells, 5 µg of IVT RNA of each chain of the NY-ESO-1 TCR or 10 µg of IVT RNA of anti-CD19- ζ CAR was added into the mixtures.

Western blots

Pellets of cells were lysed for 30 min in radioimmunoprecipitation (RIPA) buffer (Cell Signaling Technology, Danvers, MA) with a protease-phosphatase inhibitor cocktail (Cell Signaling Technology). Lysates were cleared by centrifugation at 16,000×g for 10 min. Samples were mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer with NuPAGE Sample Reducing Agent (Invitrogen), boiled for 5 min, and loaded on a 4–12% Bis-Tris polyacrylamide gel (Invitrogen). Separated proteins were transferred to polyvinylidene difluoride (PVDF; Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk or 5% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) and each antibody was probed in 5% BSA–TBST. Antibodies for LAT, phospho-LAT (Y191), phosphorylated phospholipase C- γ (PLC γ) (Y783), and β -actin were purchased from Cell Signaling Technology. Identified proteins were detected with stabilized horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Cell Signaling Technology) in blocking buffer. Membranes were visualized with SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific, Barrington, IL). The bands were scanned and quantified with ImageJ (National Institutes of Health, Bethesda, MD).

Single-cell calcium signal transduction assay

To measure the cytoplasmic calcium concentration, a slightly modified version of a previously published method (June and Moore, 2004) was used. We electroporated 1 nmol of control siRNA, LAT siRNA alone, or LAT siRNA with 5 or 20 µg of IVT RNA of LAT–YFP into *in vitro*-expanded CD8⁺ T cells. After 24 hr of incubation, 2×10^6 cells were resuspended to 1 ml with culture medium containing Indo-1-AM (2 µg/ml; eBioscience, San Diego, CA) and incubated at 37°C for 30 min. The cells were washed and stimulated with soluble anti-CD3 (OKT3 clone, 24 µg/ml; eBioscience). The ratio of the two emission wavelengths (405/20 and 530/30

with a 450-nm long-pass filter) was recorded over time with an LSR II flow cytometer (BD Biosciences, Sparks, MD) equipped with a 325-nm laser. To confirm proper Indo-1 loading and the functional potential of cells, ionomycin (15 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, MO) was used. The kinetics of the data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Single-cell enzyme-linked immunospot assay

The capture antibodies and biotinylated detection antibodies for interferon (IFN)- γ , IL-2, tumor necrosis factor (TNF)- α , and IL-4 were purchased from Mabtech (Stockholm, Sweden). LAT-substituted CD4⁺ T cells were rested for 24 hr after electroporation and then quantified with a Multisizer 3 COULTER COUNTER (Beckman Coulter, Brea, CA), cell concentration was adjusted, and cells were stimulated with anti-CD3/CD28 beads at a 1:1 ratio on capture antibody-coated 96-well filtration plates (Millipore) for 16 hr. After three washes, detection antibodies were added according to the manufacturer's protocol. Two hours later, spots were detected with an avidin-biotin-peroxidase complex (Mabtech, Nacka Strand, Sweden) and 1-Step NBT/BCIP (Thermo Fisher Scientific) substrate (NBT nitroblue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine salt). Spot-forming cells (SFCs) were objectively quantified with an AID iSpot FluoroSpot reader system (AID, Strassberg, Germany).

Intracellular cytokine staining

For intracellular cytokine staining, LAT-substituted CD4⁺ T cells were stimulated for 4 hr with immobilized anti-CD3 (5 $\mu\text{g}/\text{ml}$) or phorbol myristate acetate (PMA, 1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and ionomycin (1 μM ; Sigma-Aldrich), adding GolgiStop (BD Biosciences) at 1 hr poststimulation. These cells were treated with a FIX & PERM kit (Invitrogen) and stained with eFluor 450-conjugated anti-IFN- γ (eBioscience) and phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-IL-4 (eBioscience) according to the manufacturer's protocol. Cytometric data were collected with the LSR II and analyzed with FlowJo software.

Cellular proliferation assay

After 24 hr of incubation, LAT-substituted CD4⁺ T cells were stained with PKH26 (Sigma-Aldrich) according to the manufacturer's protocol and cultured on an anti-CD3 antibody-coated plate for 72 hr. PKH26 expression levels of these cells were measured with a FACSCalibur (BD Biosciences) and mean *T(X)* between 0 and 72 hr for each cell population was calculated, using the population comparison mode in the FlowJo program as previously published (Roederer *et al.*, 2001; Munson, 2010). *T(X)* is a statistic which provides an indication of the probability with which two distributions between 0 and 72 hr are different. Higher values of *T(X)* reflect more proliferation.

Flow-based cytotoxicity assay

Flow-based cytotoxicity assays were performed as previously described (Cao *et al.*, 2010; Zhao *et al.*, 2010). NY-ESO-1- and CD19-coexpressing target cells were generated by lentiviral transduction (Carpenito *et al.*, 2009; Milone *et al.*,

2009). To express the green fluorescent protein (GFP)-NY-ESO-1 fusion protein in Nalm-6 cells (human B cell leukemia, CD19+HLA-A2+; DSMZ, Braunschweig, Germany), the parental Nalm-6 line was transduced with GFP-NY-ESO-1 to obtain Nalm6-ESO. As a negative control, K562 cells (chronic myeloid leukemia, NY-ESO-1⁻CD19⁻; American Type Culture Collection [ATCC], Manassas, VA) were transduced to express mesothelin (K562-Meso).

Nalm6-ESO and K562-Meso cells were mixed at a 1:1 ratio and 100- μl volumes (5×10^4 cells) were aliquoted per well into 96-well round-bottom plates and then mixed with 100 μl of effector cells at several effector-to-target (*E:T*) ratios. After 4 hr of incubation, these cells were stained with anti-CD3-allophycocyanin (APC) (eBioscience) and 7-aminoactinomycin D (7AAD; BD Biosciences), and fluorescence was measured with the FACSCalibur (BD Biosciences). Specific lysis was calculated as the ratio of live Nalm6-ESO (CD3⁻7AAD⁻GFP⁺) cells and live K562-Meso (CD3⁻7AAD⁻GFP⁻) cells with FlowJo software. Result were normalized with six wells of target cells without effector cells.

CD107a mobilization assay

The CD107a mobilization assay was performed as described (Cao *et al.*, 2010). One hundred microliters of effector and target cells was plated in 96-well round-bottom plates at a ratio of 1:1, 2:1, or 4:1 (1, 2, or 4×10^5 effectors + 10^5 targets). Ten microliters of PE-Cy5-labeled anti-CD107a antibody (eBioscience) was added to each cell mixture and incubated at 37°C for 1 hr before adding GolgiStop. After an additional 2.5 hr of incubation, these cells were stained with anti-CD8-APC-Cy7 (BD Biosciences) and levels of surface CD107 were determined by flow cytometry, using the LSR II.

Statistical analysis

For statistical evaluations, Student *t* tests were performed between two groups of homoscedastic samples, and the samples with unequal variance were judged by Cochran-Cox.

Results

Replacement of endogenous LAT with ubiquitylation-resistant LAT in primary human T cells

In one study we reported that expression of a ubiquitylation-resistant LAT mutant (LAT 2KR) in Jurkat cells was more stable than that of wild-type LAT (LAT WT) and induced the enhancement of downstream signaling, including phosphorylation and calcium flux (Balagopalan *et al.*, 2011). In addition, the previous works showed increased CD69 upregulation in CD4⁺ T cells. Here we have extended the previous observations to include CD8⁺ T cells and have now investigated the effects of LAT modification on functional assays, including cytokine production, calcium signaling, phosphorylation of signaling molecules, and importantly, cytotoxicity.

To study the effects of LAT in primary human T cells, we prepared *in vitro*-transcribed (IVT) mRNA for LAT 2KR, LAT 4YF, and wild-type LAT fused to YFP (Supplementary Fig. S1). Freshly purified CD4⁺ T cells from a healthy donor were electroporated with LAT 2KR-YFP (ubiquitylation-resistant mutant), LAT 4YF-YFP (phosphorylation-resistant mutant),

and LAT WT-YFP. LAT siRNA was concurrently electroporated to selectively knock down the endogenous LAT, because the target site of this siRNA was mutated in LAT WT-YFP, LAT 2KR-YFP, and LAT 4YF-YFP. LAT siRNA and LAT-YFP IVT mRNA-electroporated cells were lysed and analyzed by Western blot (Fig. 1A). More than 65% of endogenous LAT (36 kDa) was knocked down by LAT siRNA electroporation between 24 and 48 hr after treatment. Expression of the LAT fusion proteins was dose dependent, as 20 μ g of RNA-electroporated cells expressed LAT-YFP (64 kDa) at higher levels than 10 μ g of RNA-electroporated cells, and these differences in expression were sustained for at least 48 hr. LAT expression was confirmed by measurement of YFP expression by flow cytometry (Fig. 1B and C, and Supplementary Fig. S2). expression of LAT 2KR and LAT WT-YFP fusion proteins was comparable. At 24 hr after electroporation, the YFP-positive frequencies were more than 90% in all groups of LAT-YFP-electroporated cells, and these

frequencies were continued by 48 hr. Thereafter, expression gradually decreased and was undetectable on day 6. There was no significant difference between LAT 2KR expression and LAT WT expression for 48 hr; however, the YFP expression of LAT 2KR-electroporated cells was higher than that of LAT WT from 72 to 96 hr after electroporation (Supplementary Fig. S2), confirming previous data in Jurkat cells that the LAT 2KR protein was more stable (Balagopalan *et al.*, 2011). Endogenous LAT expression had almost recovered to baseline by 72 hr after LAT siRNA electroporation.

Conditions were also established to express the altered LAT proteins in *in vitro*-expanded CD8⁺ T cells, prepared as described in Materials and Methods. In cultured CD8⁺ T cells, endogenous LAT was significantly knocked down ($p < 0.01$) and YFP-fused LAT was expressed ($p < 0.01$) (Fig. 1D and E). There was no significant difference between the expression of LAT 2KR and LAT WT. On the basis of these results, endogenous LAT could be efficiently substituted

FIG. 1. Substitution of endogenous linker for activation of T cells (LAT) by ubiquitylation-resistant LAT in primary T cells. (A) Expression of LAT small interfering RNA (siRNA) and yellow fluorescent protein (YFP)-fused LAT 2KR, 4YF, or wild-type (WT) *in vitro*-transcribed (IVT) RNA-electroporated, freshly isolated CD4⁺ T cells was analyzed by Western blot 24 or 48 hr after electroporation. Control siRNA (1 nmol) was used for negative control cells (first lane). (B and C) YFP expression of cells in (A) was confirmed by flow cytometry. The histogram and mean fluorescence intensity (MFI) are shown. (D and E) CD8⁺ T cells were stimulated with anti-CD3/CD28 beads for 10 days. After 10 days of culture, the *in vitro*-expanded cells were harvested and cryopreserved, and later these cells were thawed and incubated for 24 hr before electroporation. LAT expression of LAT siRNA and YFP-fused LAT 2KR, WT, and 4YF IVT RNA-electroporated CD8⁺ cells was analyzed by Western blot 24 hr after electroporation. (E) On the basis of the Western blot analysis of LAT siRNA and LAT-YFP RNA-electroporated CD8⁺ T cells 24 hr after electroporation, relative signal intensities of LAT were calculated. Averages and standard deviations (SD) from three different donors are shown. Statistical differences are presented with asterisks: * $p < 0.01$ (Cochran-Cox); ** $p < 0.01$ (Student *t* test).

with LAT 2KR for at least 48 hr after electroporation when 20 μ g of RNA of LAT-YFP was used. These results were confirmed in CD8⁺ cells from three different donors. In subsequent experiments, the above-described conditions were used to assess the effects of the various LAT mutants on T cell signaling and function.

Expression of ubiquitylation-resistant LAT results in enhanced T cell signaling

The T cell signaling of LAT-substituted cells was assessed in CD8⁺ T cells electroporated with YFP-fused LAT 2KR or WT IVT mRNA together with LAT siRNA. The cells were stimulated with anti-CD3/CD28 magnetic beads 24 hr after electroporation, and were lysed 10 or 30 sec after stimulation. The bands of phospho-PLC γ^{783} , phospho-LAT¹⁹¹, and total LAT are shown in Fig. 2A. The phosphorylation LAT was elevated in both LAT 2KR- and LAT WT-expressing

cells at 10 sec, and the relative phosphorylation of LAT 2KR was higher than that of LAT WT. Similarly, the phosphorylation of PLC γ in LAT 2KR-expressing cells was $\sim$ 4-fold higher than in LAT WT-expressing cells, and the phosphorylation was sustained in LAT-2KR-expressing cells in contrast to the transient signal in LAT WT cells. The kinetics of phosphorylation were further assessed by comparison of the tyrosine phosphorylation band intensities to that of LAT-YFP (Fig. 2B). Endogenous LAT was confirmed to be knocked down.

The augmented and sustained phosphorylation of PLC γ suggested that calcium signaling might be augmented in cells expressing LAT 2KR. To test this notion, we next measured calcium flux in single cells after stimulation with soluble anti-CD3 antibody, and the changes in mean intracellular free calcium concentration are shown in Fig. 2C and D. Cells expressing LAT 2KR had an augmented peak calcium signal compared with cells expressing LAT WT. The

FIG. 2. Enhanced T cell signaling of LAT-2KR-substituted CD8⁺ T cells after T cell receptor (TCR) and CD28 stimulation. (A) Phosphorylation of downstream molecules in LAT-substituted *in vitro*-expanded CD8⁺ T cells was analyzed by Western blot at three time points (0, 10, and 30 sec) after anti-CD3/CD28 bead stimulation, and the values of phospho-LAT¹⁹¹/LAT-YFP and phospho-PLC γ^{783} /LAT-YFP were calculated (B). (C and D) LAT 2KR- and LAT WT-substituted CD8⁺ T cells were stained with Indo-1, and intracellular free ionized calcium concentrations were measured by flow cytometry after stimulation with anti-CD3 antibody (triangle). YFP expression of these cells is shown in (C).

effect was dose dependent, in that cells electroporated with 20 μ g of 2KR had more pronounced calcium signaling compared with cells electroporated with 5 μ g of 2KR (Fig. 2D, middle and bottom).

Unexpectedly, we observed that cells with knocked-down endogenous LAT had an elevation of baseline calcium and an attenuated calcium signal after anti-CD3 stimulation (Fig. 2D, top). Similarly, cells with knocked-down endogenous

LAT and reconstituted with the phosphorylation-defective LAT 4YF also had constitutive elevation of calcium and a blunted calcium flux after TCR stimulation. In contrast, control siRNA-electroporated cells had the same resting calcium concentration as mock-electroporated T cells. Thus primary T cells expressing the LAT 2KR protein have increased tyrosine phosphorylation and calcium signaling compared with T cells expressing LAT WT.

FIG. 3. Increased frequency of helper T cell type 1 (Th1) cytokine secretion by CD4⁺ T cells expressing LAT ubiquitylation mutants. **(A)** Single-cell ELISPOT assay with LAT-substituted freshly isolated CD4⁺ T cells from donor 1 was performed after 16 hr of stimulation with anti-CD3/CD28 beads (solid columns) or no stimulation (shaded columns). The average and SD of three wells are shown. Statistically significant differences are indicated by asterisks: * $p < 0.001$; ** $p < 0.01$ (Student t test). **(B)** Intracellular cytokine staining of LAT-substituted CD4⁺ T cells from three different donors was measured by flow cytometry after 4 hr of stimulation with immobilized anti-CD3 antibodies. The frequency of cells expressing IFN- γ (solid columns) or IL-4 (shaded columns) is plotted.

Ubiquitylation-resistant LAT augments Th1 cytokine secretion

Studies have shown that mice expressing a tyrosine phosphorylation-defective form of LAT develop lymphoproliferative disorders involving polyclonal T cells that produced high amounts of Th2 cytokines (Malissen *et al.*, 2005). Therefore, we evaluated the effect of the LAT 2KR construct on the cytokine-producing profiles of CD4⁺ T cells by single-cell ELISPOT assay (Fig. 3A and Supplementary Fig. S3). After stimulation with anti-CD3 and anti-CD28, the frequency of IFN- γ -secreting cells in LAT 2KR-substituted cells was significantly increased compared with that of cells expressing LAT WT ($p < 0.01$). Similarly, the frequency of IL-2 and TNF- α spot-forming cells was also increased in T cells expressing LAT 2KR. Consistent with a Th1 bias, IL-4-secreting cells were decreased in LAT 2KR-substituted CD4⁺ cells ($p < 0.001$). In contrast, in LAT knockdown cells the frequency of IFN- γ secretion was less than that of control siRNA-electroporated cells. IL-2 and TNF- α spot-forming cells were also attenuated in LAT knockdown cells. As in LAT-deficient mice (Mingueneau *et al.*, 2009), we found that the frequency of IL-4-secreting cells was increased in LAT knocked-down T cells.

We used flow cytometry to measure IFN- γ - and IL-4-producing cells from three different donors by intracellular cytokine staining, as shown in Fig. 3B; the ELISPOT assay in Fig. 3A was performed on cells from donor 1. In all donors, more IFN- γ -positive cells were detected after anti-CD3 stimulation in LAT 2KR-substituted cells than in LAT WT-

substituted cells. And in donor 1, fewer IL-4-positive cells were observed in LAT 2KR-expressing cells, whereas in donors 2 and 3 the frequency of IL-4-secreting cells was unchanged. In contrast, after pharmacological stimulation with PMA and ionomycin there was no difference in the frequency of cells secreting IFN- γ (Supplementary Fig. S4), consistent with a requirement for TCR stimulation for the enhanced function of cells expressing LAT 2KR. Last, cells expressing the tyrosine phosphorylation-deficient LAT 4YF molecule had impaired Th1 cytokine secretion, similar to LAT knockdown cells (Fig. 3A).

Effects of ubiquitylation-resistant LAT on CD4⁺ cell proliferation and cytokine secretion

In the above-described experiments, the immediate effects of LAT knockdown or overexpression were assessed. We next determined whether manipulation of LAT would have an impact on T cell proliferation and function. Freshly isolated CD4⁺ T cells were electroporated, rested for 24 hr, and stained with PKH26. These cells were stimulated with immobilized OKT3 for 72 hr and the dilution of PKH26 was measured (Fig. 4A). There were no significant differences in the proliferation of T cells expressing 2KR, 4YF, or WT LAT (Fig. 4B). However, when these cells were restimulated with PMA and ionomycin to evaluate Th1 and Th2 polarization, there was a robust increase in IFN- γ and a decrease in IL-4 in cells expressing LAT 2KR compared with LAT WT or LAT 4YF cells (Fig. 4C). These results suggest that expression of ubiquitylation-resistant LAT biases cells toward Th1

FIG. 4. Cellular proliferation and Th1 differentiation of LAT-substituted CD4⁺ T cells. **(A and B)** LAT-substituted CD4⁺ T cells were stained with PKH26 at 24 hr after electroporation and cultured on an anti-CD3 antibody-coated plate for 72 hr. **(A)** Histogram of PKH26 expression from one donor; **(B)** mean proliferative index [$T(X)$ plus SD] from three different donors. NS, not significant. **(C)** LAT-substituted CD4⁺ T cells were cultured on anti-CD3 antibody-coated plates for 72 hr, and then the cytokine producing profiles were evaluated by intracellular staining after a 4-hr stimulation in the presence of phorbol myristate acetate (PMA) and ionomycin.

differentiation. Importantly, cells expressing LAT 2KR do not have spontaneous proliferation or constitutive cytokine secretion (Fig. 4C).

Ubiquitylation-resistant LAT augments TCR-mediated antitumor activity in vitro

The observation that ubiquitylation-resistant LAT augments signaling and cytokine secretion in mature T cells suggested that this strategy might enhance the potency of redirected T cells. To test this hypothesis we used the 1G4 TCR that we have previously shown to redirect T cells to recognize NY-ESO-1^{157–165} in the context of HLA-A2 (Zhao *et al.*, 2006). mRNA encoding the α and β chains of the 1G4 TCR was coelectroporated into *in vitro*-expanded resting CD8⁺ T cells along with LAT siRNA and LAT-YFP. The anti-NY-ESO-1-specific TCR was expressed in more than 90% of cells and there was no significant difference in expression due to the introduction of the various LAT mutants (Supplementary Fig. S5A). The cytotoxic activities of these cells against Nalm6-ESO-1 targets expressing HLA-A2 and

NY-ESO-1 were analyzed by a flow cytometry-based cytotoxic assay (Fig. 5A). Importantly, the cytotoxicity of LAT 2KR-substituted cells was significantly augmented compared with LAT WT-substituted cells and with T cells expressing endogenous LAT. In contrast, the cytotoxicity of LAT knockdown cells and LAT 4YF-substituted cells was inhibited compared with redirected T cells expressing endogenous LAT.

To further characterize the function of the NY-ESO-1 TCR we assessed the mobilization of CD107a (Fig. 5B). CD8⁺ T cells expressing the anti-NY-ESO-1 TCR and the engineered LAT molecules were coincubated with 624.38 melanoma cells (HLA-A2⁺NY-ESO-1⁺) or with 526 melanoma cells (HLA-A2⁺NY-ESO-1⁻). LAT 2KR T cells had augmented CD107a translocation in comparison with all other cell populations. The effector activity was specific for NY-ESO-1, and CD107a was induced in a tumor dose-dependent manner. The cells with endogenous LAT knockdown had a modest inhibition of CD107a translocation compared with redirected T cells reconstituted with LAT WT or with T cells electroporated only with the TCR

FIG. 5. Enhanced cytotoxicity of TCR and chimeric antigen receptor (CAR)-redirected CD8⁺ T cells expressing LAT ubiquitylation mutants. (A, C, and D) Flow-based cytotoxicity assays against NY-ESO-1-transduced Nalm-6 target cells were performed after a 4-hr incubation with effector cells. Effector cells were anti-NY-ESO-1 TCR expressed in CD8⁺ T cells with endogenous LAT knockdown (A); anti-NY-ESO-1 TCR expressed in CD8⁺ T cells with the indicated LAT mutants without endogenous LAT knockdown (C); and anti-CD19- ζ CAR expressed in CD8⁺ T cells with endogenous LAT knockdown (D). The average $\pm$ SD of triplet samples was plotted. Statistically significant differences are indicated by asterisks: * p < 0.01 (Student *t* test); ** p < 0.05 (Cochran-Cox). (B) CD107a mobilization assay against 624.38 (NY-ESO-1⁺HLA-A2⁺) or 526 (NY-ESO-1⁻HLA-A2⁺) melanoma target cells was performed with anti-NY-ESO-1 TCR-expressing LAT-substituted T cells. CD8⁺ gated cells are shown.

mRNA. These results are consistent with the previous cytotoxicity assays.

We next explored whether it was necessary to knock down endogenous LAT in order to augment the cytotoxic function of T cells. IVT mRNA directing the expression of the NY-ESO-1 TCR α/β and LAT-YFP were electroporated into CD8⁺ T cells without LAT siRNA. The introduction of TCR mRNA did not impair expression of the LAT fusion proteins (Supplementary Fig. S5B). Immunoblot analysis confirmed that the LAT fusion proteins could be expressed in T cells at levels exceeding endogenous LAT (Supplementary Fig. S5C), and flow cytometry indicated that more than 90% of the cells expressed the fusion proteins (Supplementary Fig. S5D). LAT 2KR-overexpressing T cells showed significantly increased cytotoxicity (Fig. 5B). This effect was specific for the ubiquitylation-resistant form of LAT, as LAT WT- or LAT 4YF-overexpressing cells did not exhibit any enhancement compared with control cells that were expressing the specific TCR without LAT-YFP. The finding that the knockdown of endogenous LAT is not required for the therapeutic strategy using LAT 2KR has important practical implications.

Ubiquitylation-resistant LAT augments CAR mediated antitumor activity in vitro

Last, we investigated whether T cells engineered to express LAT 2KR and a chimeric antigen receptor (CAR) would have enhanced functional activity. For these experiments we used the anti-CD19- ζ CAR (Milone *et al.*, 2009). To simplify the interpretation of this approach we used a CD3 ζ -only CAR, because the signaling via costimulatory molecules would complicate the evaluation of the various LAT constructs. CD8⁺ T cells were electroporated with 10 μ g of anti-CD19- ζ CAR mRNA together with LAT siRNA and LAT-YFP constructs. The CAR was expressed at the cell surface in more than 75% of cells and there were no significant differences in the efficiency of expression between cells expressing LAT 2KR, LAT WT, or LAT 4YF (Supplementary Fig. S5E). When tested on the Nalm-6 tumor line, the CD19-specific cytotoxicity of T cells expressing LAT 2KR was significantly augmented (Fig. 5D). In contrast, the cytotoxicity of LAT knockdown cells and LAT 4YF-substituted cells was attenuated. Thus, T cells with engineered LAT have the potential to augment the therapeutic efficacy of CAR T cells.

Discussion

Here we report the potential of modified LAT to improve the effector functions of primary postthymic human T cells. In one study Balagopalan and colleagues (2011) showed that expression of ubiquitylation-resistant LAT 2KR increased signaling in human T leukemia cells by two mechanisms: more LAT was expressed and LAT 2KR was more potent in a molecule-to-molecule comparison. We find here that electroporation of mRNA results in efficient expression of LAT 2KR and that this results in increased signaling and function of CD4⁺ and CD8⁺ human T cells. We found that in primary T cells, the phosphorylation of wild-type LAT was rapid in onset and transient, whereas phosphorylation of 2KR LAT was augmented and sustained. Because phosphorylation and ubiquitylation reactions

occur within seconds (Fig. 2A; and Pierce *et al.*, 2009), it is likely that once phosphorylated, wild-type LAT becomes rapidly ubiquitylated. Ubiquitylation likely leads to a change in LAT localization, dephosphorylation, and finally degradation. In contrast, phosphorylated 2KR LAT persists because it is resistant to ubiquitylation.

Stimulation of CD4⁺ T cells that express LAT 2KR induced a pattern of cytokine secretion consistent with Th1 differentiation, whereas cytokine secretion in LAT knockdown cells was consistent with a Th2 pattern of differentiation. Signaling through the TCR can regulate Th1 and Th2 differentiation (Constant *et al.*, 1995; Nakayama and Yamashita, 2010). Previous studies have shown that LAT has a role in T cell development in the balance of Th1 and Th2 differentiation (Malissen *et al.*, 2005).

We also found that expression of LAT 2KR increased the activation of CD8⁺ T cells after tumor antigen presentation. It was notable that cytotoxicity was enhanced in LAT 2KR cells, and that this augmentation was observed not only after antigen presentation to MHC class I-restricted TCRs but also after antigen stimulation of T cells expressing chimeric antigen receptors. The enhanced cytotoxicity was specific for T cells expressing the ubiquitin-resistant form of LAT.

An unexpected finding in our studies was that LAT knockdown T cells had an elevated basal level of intracellular calcium concentration. This suggests that one function of LAT is to negatively regulate calcium homeostasis in quiescent cells. Others have previously reported that LAT can have negative regulatory function in mouse T cells (Mingueneau *et al.*, 2009). In lymph nodes, there is evidence of continuous "tonic" signaling through the TCR, and that this is required to maintain antigen responsiveness (Hochweller *et al.*, 2010). Consistent with a role for LAT in basal T cell signaling, imaging studies have shown that membrane domains of both LAT and TCR ζ significantly overlap under both activating and nonactivating conditions (Sherman *et al.*, 2011). Our studies suggest that LAT may play a role in regulating the tonic resting state of the lymphocyte (Hochweller *et al.*, 2010) as well as the activated state during antigen stimulation (Lillemeier *et al.*, 2010).

In these initial studies we have used transient expression of modified LAT to evaluate the potential of LAT 2KR for potential clinical applications. This would provide safety but may limit the potency in the clinic. Further studies will be required to evaluate the safety and efficacy of engineered T cells that are permanently modified to express LAT 2KR. Thus a limitation of the present studies is that the safety of this approach with constitutive expression of ubiquitin-resistant LAT remains unknown. Another limitation of this approach is that it is possible that augmented LAT signaling could result in the induction of activation-induced cell death (AICD). Our results show that the enhanced signaling mediated by LAT does not decrease proliferation during the first 3 days of stimulation; however, it is possible that during longer term stimulation this could result in the induction of AICD and impaired proliferation of LAT-engineered T cells *in vivo*.

Ubiquitylation-resistant LAT is a novel approach to enhance T cell signaling and has the potential to augment effector T cell function for adoptive cell therapy. We found that the function of T cells expressing an MHC class I-restricted TCR was enhanced by ubiquitin-resistant LAT. It is possible

that this approach might improve the effector functions of T cells expressing low-affinity TCRs that are typical of many tumor-associated antigens (Kessels *et al.*, 2001; Zehn *et al.*, 2009). Last, the combination of an antigen-specific CAR with ubiquitylation-resistant LAT could be considered an example of a “next-generation CAR” that has promise to further enhance the effector functions of engineered T cells for cancer and chronic viral infections.

Acknowledgments

The authors thank Carmine Carpenito, Michael Kalos, and Caleph Wilson (University of Pennsylvania) and Shinichiro Motohashi (Department of Immunology and Thoracic Surgery, Graduate School of Medicine, Chiba University, Chiba, Japan) for advice; and members of the Human Immunology Core (Abramson Cancer Center) for providing lymphocytes. This work was funded by the National Institutes of Health (NIH) Common Fund Nanomedicine program (PN2 EY016586), and this research was supported, in part, by the Intramural Research Program of the NIH, NCI, CCR (L.B. and L.E.S.).

Author Disclosure Statement

Dr. Balagopalan and Dr. Samelson have filed a patent on behalf of the U.S. Government. All other authors have no conflict of interest.

References

- Balagopalan, L., Ashwell, B.A., Bernot, K.M., *et al.* (2011). Enhanced T-cell signaling in cells bearing linker for activation of T-cell (LAT) molecules resistant to ubiquitylation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2885–2890.
- Barrett, D.M., Zhao, Y., Liu, X., *et al.* (2011). Treatment of advanced leukemia in mice with mRNA engineered T cells. *Hum. Gene Ther.* 22, 1575–1586.
- Bonini, C., Brenner, M.K., Heslop, H.E., and Morgan, R.A. (2011). Genetic modification of T cells. *Biol. Blood Marrow Transplant.* 17, S15–S20.
- Cao, L.F., Krymskaya, L., Tran, V., *et al.* (2010). Development and application of a multiplexable flow cytometry-based assay to quantify cell-mediated cytotoxicity. *Cytometry A* 77, 534–545.
- Carpenito, C., Milone, M.C., Hassan, R., *et al.* (2009). Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc. Natl. Acad. Sci. U.S.A.* 106, 3360–3365.
- Chiang, J.Y., Jang, I.K., Hodes, R., and Gu, H. (2007). Ablation of Cbl-b provides protection against transplanted and spontaneous tumors. *J. Clin. Invest.* 117, 1029–1036.
- Constant, S., Pfeiffer, C., Woodard, A., *et al.* (1995). Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *J. Exp. Med.* 182, 1591–1596.
- Friberg DD, Bryant JL, and Whiteside TL. (1996). Measurements of Natural Killer (NK) Activity and NK-Cell Quantification. *Methods* 9, 316–26.
- Greenberg, P.D., and Riddell, S.R. (1999). Deficient cellular immunity—finding and fixing the defects. *Science* 285, 546–551.
- Hochweller, K., Wabnitz, G., Samstag, Y., *et al.* (2010). Dendritic cells control T cell tonic signaling required for responsiveness to foreign antigen. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5931.
- Horejsi, V., Zhang, W., and Schraven, B. (2004). Transmembrane adaptor proteins: Organizers of immunoreceptor signalling. *Nat. Rev. Immunol.* 4, 603–616.
- Johnson, L.A., Morgan, R.A., Dudley, M.E., *et al.* (2009). Gene therapy with human and mouse T cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 114, 535–546.
- June, C.H., and Moore, J.S. (2004). Measurement of intracellular ions by flow cytometry. *Curr. Protoc. Immunol.* Chapter 5, Unit 5.5.
- Kalos, M., Levine, B.L., Porter, D.L., *et al.* (2011). T cells expressing chimeric receptors establish memory and potent antitumor effects in patients with advanced leukemia. *Sci. Transl. Med.* 3, 95ra73.
- Kessels, H.W., Wolkers, M.C., Van Den Boom MD, *et al.* (2001). Immunotherapy through TCR gene transfer. *Nat. Immunol.* 2, 957–961.
- Kohn, D.B., Dotti, G., Brentjens, R., *et al.* (2011). CARs on track in the clinic: Report of a meeting organized by the Blood and Marrow Transplant Clinical Trials Network (BMT CTN) Subcommittee on Cell and Gene Therapy. *Washington D.C.*, May 18, 2010. *Mol. Ther.* 19, 432–438.
- Krause, A., Guo, H.F., Latouche, J.B., *et al.* (1998). Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. *J. Exp. Med.* 188, 619–626.
- Lillemeier, B., Mörtelmaier, M., Forstner, M., *et al.* (2010). TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nat. Immunol.* 11, 90–96.
- Malissen, B., Aguado, E., and Malissen, M. (2005). Role of the LAT adaptor in T-cell development and Th2 differentiation. *Adv. Immunol.* 87, 1–25.
- Milone, M.C., Fish, J.D., Carpenito, C., *et al.* (2009). Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy *in vivo*. *Mol. Ther.* 17, 1453–1464.
- Mingueneau, M., Roncagalli, R., Gregoire, C., *et al.* (2009). Loss of the LAT adaptor converts antigen-responsive T cells into pathogenic effectors that function independently of the T cell receptor. *Immunity* 31, 197–208.
- Munson, M.E. (2010). An improved technique for calculating relative response in cellular proliferation experiments. *Cytometry A* 77, 909–910.
- Nakayama, T., and Yamashita, M. (2010). The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. *Semin. Immunol.* 22, 303–309.
- Paulos, C.M., Carpenito, C., Plesa, G., *et al.* (2010). The inducible costimulator ICOS is critical for the development of human TH17 cells. *Sci. Transl. Med.* 2, 55–78.
- Paz, P.E., Wang, S., Clarke, H., *et al.* (2001). Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells. *Biochem. J.* 356, 461–471.
- Pierce, N.W., Kleiger, G., Shan, S.O., and Deshaies, R.J. (2009). Detection of sequential polyubiquitylation on a millisecond timescale. *Nature* 462, 615–619.
- Riese, M.J., Grewal, J., Das, J., *et al.* (2011). Decreased diacylglycerol metabolism enhances ERK activation and augments CD8⁺ T cell functional responses. *J. Biol. Chem.* 286, 5254–5265.
- Roederer, M., Moore, W., Treister, A., *et al.* (2001). Probability binning comparison: A metric for quantitating multivariate distribution differences. *Cytometry* 45, 47–55.

- Roncagalli, R., Mingueneau, M., Gregoire, C., *et al.* (2010). Lymphoproliferative disorders involving T helper effector cells with defective LAT signalosomes. *Semin. Immunopathol.* 32, 117–125.
- Sadelain, M., Riviere, I., and Brentjens, R. (2003). Targeting tumours with genetically enhanced T lymphocytes. *Nat. Rev. Cancer* 3, 35–45.
- Schumacher, T.N. (2002). T-cell-receptor gene therapy. *Nat. Rev. Immunol.* 2, 512–519.
- Shen, S., Chuck, M.I., Zhu, M., *et al.* (2010). The importance of LAT in the activation, homeostasis, and regulatory function of T cells. *J. Biol. Chem.* 285, 35393–35405.
- Sherman, E., Barr, V., Manley, S., *et al.* (2011). Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. *Immunity* 35, 705–720.
- Simeoni, L., Lindquist, J.A., Smida, M., *et al.* (2008). Control of lymphocyte development and activation by negative regulatory transmembrane adapter proteins. *Immunol. Rev.* 224, 215–228.
- Zehn, D., Lee, S.Y., and Bevan, M.J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458, 211–214.
- Zhang, W., Sloan-Lancaster, J., Kitchen, J., *et al.* (1998). LAT: The ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell* 92, 83–92.
- Zhang, W., Tribble, R.P., Zhu, M., *et al.* (2000). Association of Grb2, Gads, and phospholipase C- γ 1 with phosphorylated LAT tyrosine residues: Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J. Biol. Chem.* 275, 23355–23361.
- Zhao, Y., Zheng, Z., Robbins, P.F., *et al.* (2005). Primary human lymphocytes transduced with NY-ESO-1 antigen-specific TCR genes recognize and kill diverse human tumor cell lines. *J. Immunol.* 174, 4415–4423.
- Zhao, Y., Zheng, Z., Cohen, C.J., *et al.* (2006). High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. *Mol. Ther.* 13, 151–159.
- Zhao, Y., Moon, E., Carpenito, C., *et al.* (2010). Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res.* 70, 9062–9072.
- Zhu, M., Janssen, E., and Zhang, W. (2003). Minimal requirement of tyrosine residues of linker for activation of T cells in TCR signaling and thymocyte development. *J. Immunol.* 170, 325–333.

Address correspondence to:
Dr. Carl H. June
University of Pennsylvania
3400 Civic Center Boulevard
8th Floor, Room 08-123
Philadelphia, PA 19104-5156

E-mail: cjune@exchange.upenn.edu

Received for publication June 25, 2012;
accepted after revision September 7, 2012.

Published online: September 21, 2012.