Supplementary Material

Methods

Overlapping PCR using LV-GM-CSF-P2A-IL-4 and LV-tWT1 as templates

For construction of LV-G242W, we introduced the interspacing foot-and-mouth disease virus 2A element (F2A) upstream of WT1 as previously described (Pincha *et al.*, 2012). The primers used in generating the interspacing F2A element were: F2A-IL-4-Reverse: 5′-GGGCCCTGGGTTTGG ACTCCACGTCTCCCGCCAACTT-3′. F2A-hWT1 forward: 5′-CCGGTGAAACAGACTTTGAATTTTGACCTTCTCAAG-3′. The structural integrity of all constructs was reconfirmed by restriction digestion and sequencing analysis of all transgenes.

Cell culture

Human embryonic kidney 293T cells were cultured in DMEM (Invitrogen), with 10% FBS and penicillin (100 U/mL) and streptomycin (100 mg/mL). K2A cells (K562 cells stably transfected for expression of HLA-A*02:01 [Britten *et al.*, 2002]) were cultured in RPMI with 10% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and geneticin (1 mg/mL) (Biochrom AG). K2A/tWT1 were generated after transduction of K2A cells with LV-tWT1, expanded, and cryopreserved in large batches.

A T-cell clone (CF69) containing a transgenic TCR reactive against the $WT1_{126–134}$ epitope was provided by Prof. Chiara Bonini and used as control. CF69 was expanded by co-culture with a mixture of 3 different allogeneic PBMCs, irradiated at 30 Gy at the concentration of 10^6/mL, allogeneic Rosi-EBV cell line (a kind gift from Prof. Vigo van Tendeloo) at a concentration of 0.2×10^6 cells/ml and 30 ng/mL of OKT3 antibody in 25 ml of IMDM supplemented with 10% FBS and 600 IU/ml of rhIL-2. After 3 days, OKT3 antibody is washed away, and the cells were expanded in culture for 14 days and cryopreserved.

Western blot analyses

Cell supernatants were collected, and 293T cells were treated with lysis buffer (with protease inhibitor cocktail; Bio-Rad) for 15 min on ice. Cell lysates and supernatant samples were then mixed with loading buffer, boiled at 94°C for 10 min and loaded onto SDS-PAGE gels for electrophoresis (AnykD gel, Bio-Rad). Immunodetection was performed with the following primary antibodies: rabbit anti-human GM-CSF (Peprotech), mouse anti-human IL-4 (R&D Systems), mouse anti-human-WT1 (Santa Cruz). Protein bands were visualized using appropriate secondary antibodies coupled to horseradish peroxidase and the enhanced chemi-luminescence (ECL) kit (Pierce, Thermo Scientific) according to the manufacturer's instructions.

Generation of human SmartDCs or conventional DCs

SmartDCs were generated using an established protocol (Salguero *et al.*, 2011). Briefly, $CD14^+$ cells were isolated using CD14 isolation beads (Miltenyi Biotec) and further cultured in X-VIVO 15 medium (Lonza) in the presence of recombinant human GM-CSF and IL-4 (50 ng/mL each; Cellgenix) for 8 hr before transduction. 5×10^6 $CD14^+$ monocytes were transduced with LVs in six-well plates containing 3 μg p24 equivalent/mL of each vector and 5 μg/mL protamine sulfate (American Pharmaceutical Partners, Inc.). Sixteen hr post transduction, cells were washed twice and further cultured in X-VIVO 15 medium without cytokines or cryopreserved. Conventional DCs were generated from isolated $CD14^+$ cells continuously supplemented with GM-CSF and IL-4 (50 ng/mL each) every 3 days.

Analyses of human GM-CSF and IL-4 transgene expression

Secreted human GM-CSF and IL-4 in supernatants were detected as described with commercially available ELISA kits (R&D Systems). Detection of additional human cytokines and chemokines in the supernatants of DC cultures was performed with fluorescent bead-based 14-plex Luminex assay according to the manufacturer's protocol (Invitrogen). The 14plex assay measured the following molecules: GM-CSF, IL-4, TNF-α, IL-6, IL-8, MCP-1, IL-10, IL-1β, IL-5, IL-13, IFN-γ, IL-7, IL-2, and IL-12(p70). The sensitivity limit of the assay was 3.2 pg/mL for each cytokine.

Intracellular staining for WT1 protein expression analyses

WT1 transgene expression was evaluated in KA2 and primary AML cells by intracellular staining and FACS analysis. Briefly, KA2 transduced with LV-tWT1 were permeabilized and fixed with cytofix/cytoperm solution (Becton Dickinson) according to manufacturer's instructions, followed by incubation with monoclonal mouse anti-WT1 (Santa Cruz Biotechnology, Inc.) and a secondary Dylight649 conjugated goat anti-mouse antibody (Biolegend). Cells are then washed with BD perm/wash solution (Becton Dickinson) and analyzed by flow cytometry.

Quantification of WT1 RNA

cDNA was prepared from 1 μg of RNA by RT (reverse transcription), according to the manufacturer's protocol. Briefly, random hexamers (Invitrogen) at a concentration of 25 μM and 100 U of MMLV reverse transcriptase (Invitrogen) were used in a total volume of 20 μl leading to substantial amplification. The RQ-PCR and the fluorescence measurements were done using ABI StepOnePlus™ (Life Technologies GmbH). The reaction was set up according to the protocol provided with the kit. Briefly, 5 μl of cDNA prepared from the above step was added to 20 μl of RQ-PCR mix containing 12.5 μl of Taqman® Universal PCR master mix (Applied Biosystems) with 1 μl of primers and probe mix, adjusting the volume to 20 μl with PCR grade, nuclease-free water. RQ-PCR reaction was run as follows: 50°C for 2 min (1 cycle), 95°C for 10 min (1 cycle), and 95°C for 15 sec followed by 60°C for 1 min (50 cycles). Calibration

curve was obtained using the standards provided for WT1 and ABL (housekeeping gene) with the kit. The WT1 transcripts were normalized with respect to the number of ABL transcripts amplified in the same reaction and expressed as WT1 mRNA copies per 1×10^4 copies of ABL.

Flow cytometry analyses of DCs

We used the following antibodies: PE-conjugated anti-human CD86, PerCP-conjugated anti-human HLA-DR, PE-conjugated anti human CD80, AlexaFlour 647-conjugated CCR2, PerCP-conjugated CCR5 (Becton Dickinson), or with their respective isotype controls. Cells were collected, washed once with PBS, and resuspended in PBS containing mouse IgG (50 μg/mL) on ice for 15 min followed by incubation with the corresponding monoclonal antibodies for 30 min. Cells were washed and resuspended in cell fix solution (Becton Dickinson). Cells are then analyzed using a FACSCalibur cytometer (Becton Dickinson). Acquisition was performed using CellQuest software and analyses were performed using CellQuest software (Becton Dickinson) or Flowjo software (Treestar).

Statistical analysis of the gene array expression data

Gene array analysis was performed using the GeneSpring GX 11 Software (Agilent Technologies). Data mining and the statistical analyses was performed as follows: Raw signal intensities were log2 transformed and normalized using scaling to mean expression data of all arrays. Subsequently, data was cleaned by removing data points that were not informative and near background intensity (excluding those genes from further analysis the expression intensity of which never exceeded the 20th percentile or were flagged as "absent" by Feature Extraction Software). Biological replicates were combined into groups and applied to statistical ANOVA analysis. Genes were selected for further analysis, when the obtained p-value was below 0.05 and the expression changed by more than two-fold.

Thymidine incorporation and IFN-γ ELISPOT analyses from bulk cultures

1×10^5 $CD8^+$ T cells were co-cultured with 1×10^4 SmartDCs (at 1:10 ratio) for 5 days in a 96 well-U bottomed plates. Triplicate wells were set up for each condition. On day 5, 1μCi/well of [^{3}H] thymidine was added to the wells and incubated for 18 hours at 37°C. [^{3}H] thymidine incorporation was measured on a β-scintillation counter.

For IFN-γ ELISPOT assay, *in vitro*–stimulated day-30 T cells were seeded at a density of 1×10^6 cells per well in 96-well ELISPOT plate coated with anti-human IFN-γ (Mabtech AB, Germany). As control for T-cell stimulation in ELISPOT, the CEF peptide pool containing 32 HLA class I-restricted T-cell epitopes from human cytomegalovirus, Epstein-Barr virus, and influenza virus was used. The cells were incubated with the peptides; $WT1_{126–134}$, WT1 pepmix, and CEF and incubated for 20 hours at 37°C and 5% CO_2. After incubation, cells were discarded and plates were further incubated with biotin-conjugated anti-human IFN-γ antibody followed by alkaline phosphatase-conjugated streptavidin. Plates were developed using NBT/ BCIP liquid substrate (Sigma) and analyzed with an ELISPOT reader (CTL Immunospot® analyzer) and quantified with ImmunoSpot® Software for ELISPOT Analysis (CTL-Europe GmbH).

Microcultures for T-cell stimulation and IFN-γ ELISPOT

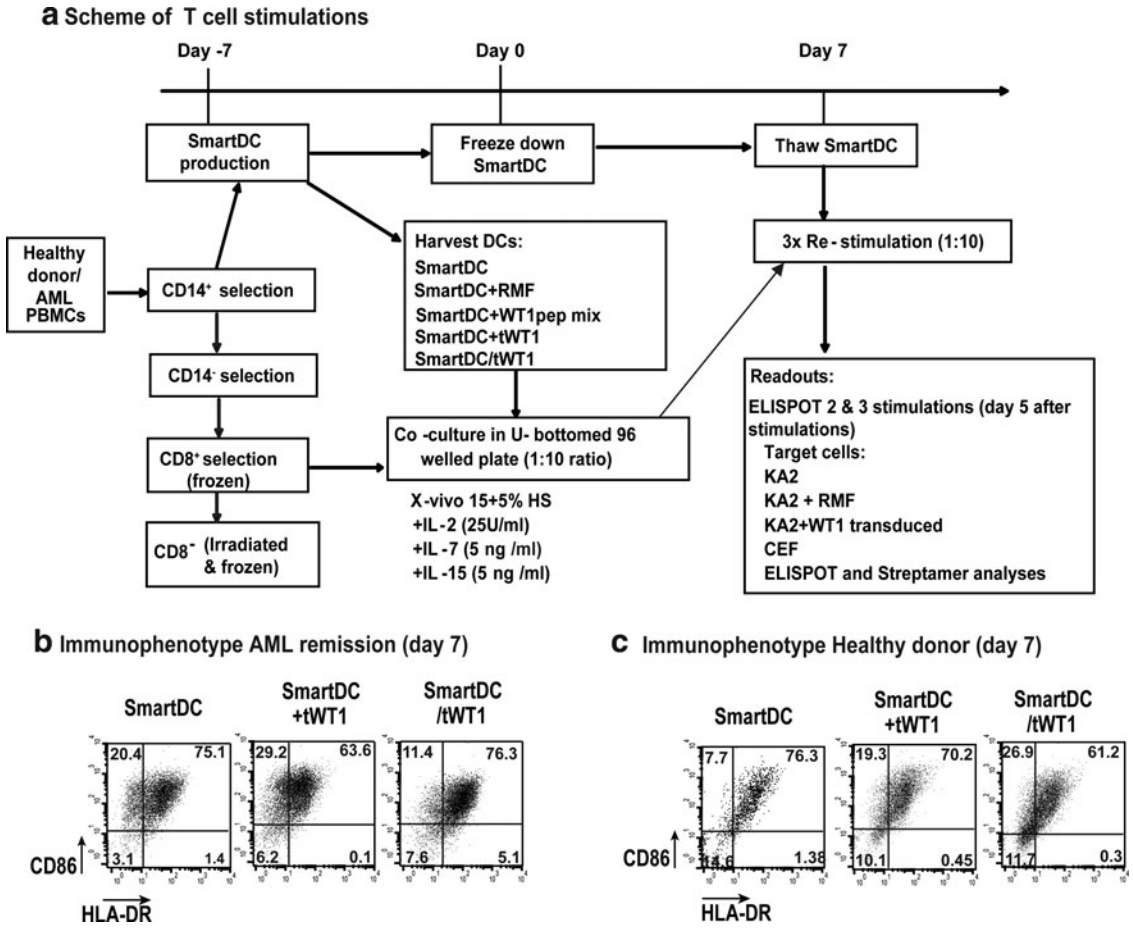
$CD8^+$ T cells were seeded into a 96-well-U bottom plate at 1×10^5 cells/well, and autologous SmartDCs were added at a 10:1 ratio (T cell:DC) (Supplementary Fig. 1a). 2×10^5 autologous feeder cells (CD14 and CD8 negative) gamma-irradiated with 40 Gy were added to the co-culture. X-VIVO 15 supplemented with 5% human AB serum (Lonza) and cytokines at following concentrations: IL-2 (25 IU /mL) (Proleukin), IL-7 (5 ng/mL), and IL-15 (5 ng/mL) (Cellgenix), was added to a final volume of 200 μl per well. Cultures were then kept at 37°C for 7 days; medium and cytokines were replenished every two days. After 7 days, the subsequent stimulation was performed after determining the T-cell number and adding appropriate numbers of DCs, always maintaining the 10:1 T:DC ratio. IFN-γ ELISPOT assay was performed as described above. 2.5×10^4 T cells were incubated with 7.5×10^4 KA2 target cells per well and incubated for 20 hours at 37°C and 5% CO_2. After incubation, plates were processed for detection and quantification of spots.

Streptamer analyses

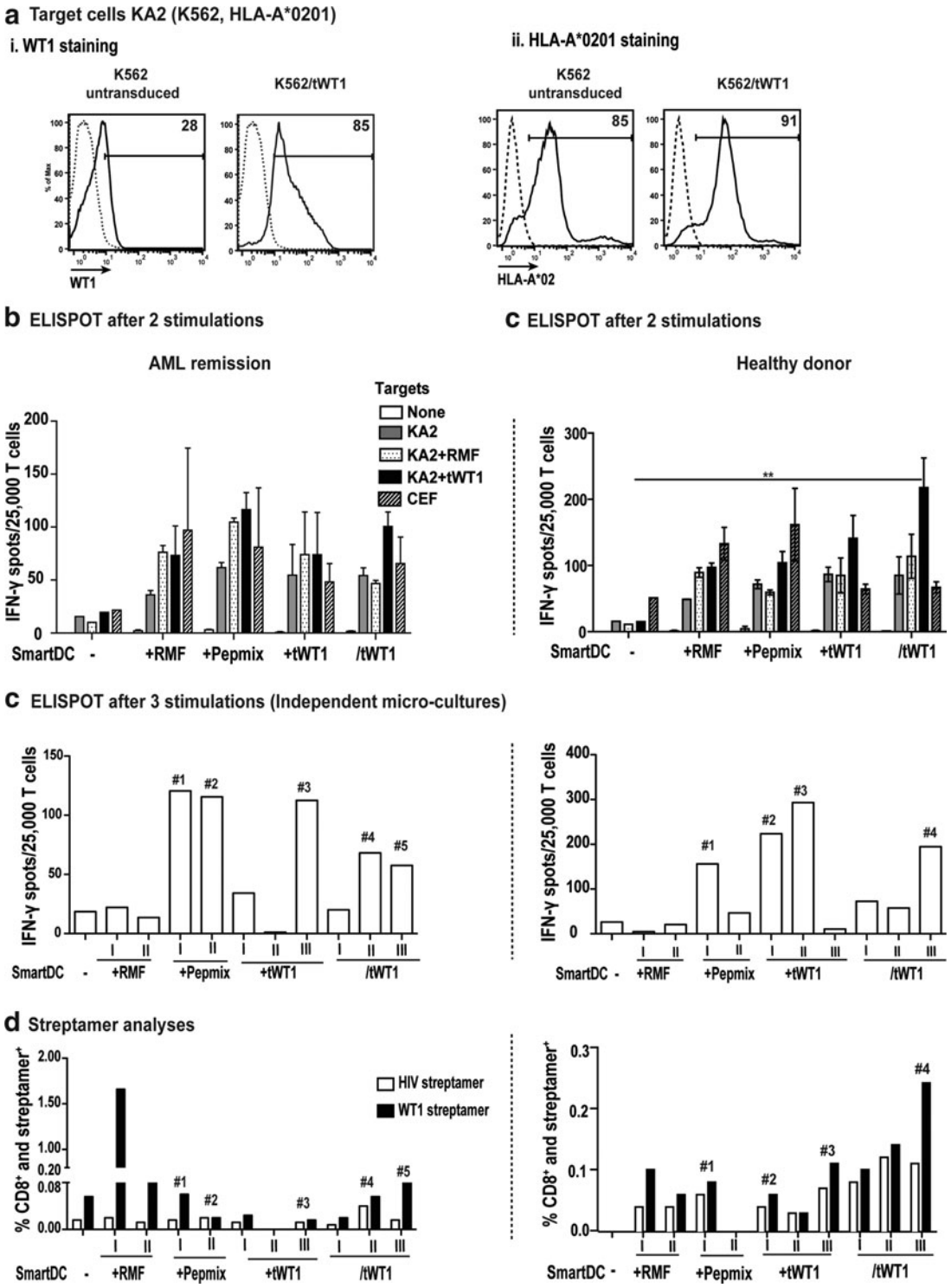
1×10^6 T cells were stained with 0.75 μg of streptactin PE and 1 μg of the WT1-reactive streptamer or a nonspecific HIV streptamer (specific for HIV-gag epitope SLYNTVATL) for 45 min in dark at 4°C in 50 μl of PBS supplemented with 0.5% BSA. The T cells were subsequently stained with FITC-conjugated anti-CD8 (Beckman Coulter) and CD3-PCy7-conjugated anti-CD3 (Biolegend GmbH) for 30 min in dark at 4°C. Cells were washed with PBS supplemented with BSA, resuspended in the same buffer, and kept on ice until acquisition. Dead cells were negatively selected after labeling with 7-AAD (Beckman Coulter). At least 1×10^5 CD3 and CD8 positive cells were acquired. Results are expressed as percentage of T cells reactive against the $WT1_{126–134}$ epitope within the $CD8^+$ cell population.

Carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity analyses

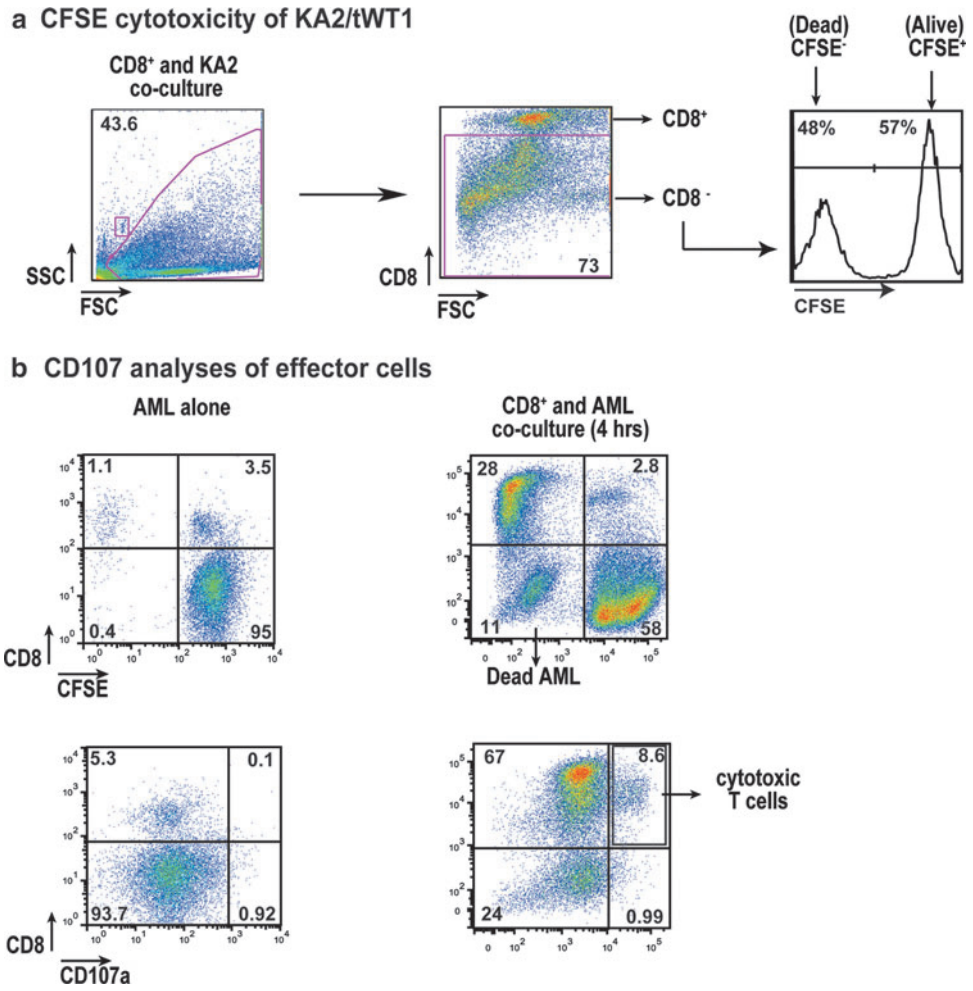
KA2/tWT1 cells were washed with PBS, resuspended at 1.0×10^7 cells/mL, labeled with 10 μM CFSE for 10 minutes at 37°C, washed and resuspended in X-VIVO-15 at 5×10^6 cells/mL. The T cells expanded with different SmartDC cultures were harvested, washed, and resuspended in X-VIVO-15 at 5×10^4 cells/mL. T cells and KA2/tWT1/ CFSE targets were mixed in various E:T ratios and incubated at 37°C and 5% CO_2 for 8 hr. After co-culture, T cells were labeled with fluorochrome-conjugated antibodies (PE-Cy7-CD8 and APC-CD3; BD Biosciences). Fluorochrome-labeled microbeads were added to facilitate the quantification of the cells by flow cytometry. The acquisition was carried out in FACS LSR-II (BD biosciences) and the results were analyzed using Flowjo software v. 7.6.4 (Treestar Inc.)



SUPPLEMENTARY FIG. 1. Microculture method for expansion of WT1 reactive T cells: **(a)** detailed scheme of T-cell stimulations performed with AML remission and healthy donor samples. Scheme depicts the various steps involved in the stimulation process, starting with SmartDC production and ending with ELISPOT and streptamer analyses after the stimulation cycles, including the intermediate steps involved in the process. **(b)** Immunophenotype of SmartDC, SmartDC+ tWT1, and SmartDC/tWT1 from both AML patient sample and healthy donor, used in the stimulation of $CD8^+$ T cells. Dot plots display HLA-DR^+ and $CD86^+$ double positive cells. Numbers indicate the percentage positive in each group.



SUPPLEMENTARY FIG. 2. Analyses of WT1-reactive $CD8^+$ T cells using KA2/tWT1 as targets. **(a)** Characterization of target cells, KA2 and KA2/tWT1: (i) WT1 expression and (ii) HLA-A2 expression in KA2 and in KA2/tWT1 respectively. The dotted line indicates the isotype control, whereas the full line indicates the WT1/HLA-A2 staining. Numbers indicate the percentage positive cells. **(b)** ELISPOT analyses after the third stimulation cycle. Stimulated $CD8^+$ T (2.5×10^4) cells harvested from each microculture and were co-cultured with target cells: KA2 alone, KA2 loaded with $WT1_{126–134}$, KA2 loaded with WT1 peptide mix, and KA2/tWT1.The average IFN-γ spots from duplicate wells per group were quantified. Bar graphs show the grouped data from independent microculture wells. White bars indicate T cells alone in each group, whereas black bars indicate T cells co-cultured with KA2/tWT1. Error bars indicate SEM. $^{**}p<0.01$ **(c)** Independent microcultures after three stimulations are shown. Bar graphs represent the data obtained from duplicate wells from each microculture condition against KA2/tWT1. The numbers on the bars indicate the microcultures corresponding to the streptamer analyses. **(d)** Streptamer analyses of independent microcultures after three stimulations. Histogram bars represent the percentage positive WT1-specific T cells. White bars represent HIV streptamer and black bars represent WT1 immuno-dominant epitope: HLA A*02:01-$WT1_{126–134}$. The numbers on the bars indicate the corresponding microcultures from ELISPOT analyses.



SUPPLEMENTARY FIG. 3. **(a)** CFSE-based cytotoxicity assay gating strategy and the dot plots and histograms depicting the mode of analyses performed to achieve the percentage specific lyses, before and after cytotoxicity. The KA2 was labeled with CFSE and used in co-culture with $CD8^+$ T cells generated from the microculture method for 6 hr. The live cells were gated on $CD8^-$ cells, which were further gated on CFSE shown in a histogram plot showing the $CFSE^+$ and $CFSE^-$ populations, an indicator of specific lytic ability of T cells against KA2/tWT1. Numbers indicate percentage $CFSE^+$ and $CFSE^-$. **(b)** Gating strategy and the mode of analyses in the CD107a degranulation assay performed on effector T cells. The primary AML targets were labeled with CFSE. The CFSE staining and the CD107a expression in the target cells were determined by FACS analyses before (left panel) and after (right panel) the 4-hr co-culture respectively.