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Functional Leukemia-associated Antigen-Specific Memory CD8$^+$ T Cells Exist in Healthy Individuals and in Patients with Chronic Myelogenous Leukemia Before and After Stem Cell Transplantation.

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ABSTRACT

Antigens implicated in the graft-versus-leukemia (GVL) effect in chronic myeloid leukemia (CML) include WT1, PR1 and BCR-ABL. To detect very low frequencies of these antigen-specific $CD8^+$T cells, we used quantitative PCR (qPCR) to measure interferon-gamma (IFN-γ) mRNA production by peptide-pulsed $CD8^+$ T cells from HLA-$A*0201^+$ healthy volunteers, and CML patients before and after allogeneic stem cell transplantation (SCT). Parallel assays using CMV pp65 tetramers demonstrated the IFN-γ copy number to be linearly related to the frequency of tetramer-binding T cells, sensitive to frequencies of 1 responding $CD8^+$ T cell/100,000 $CD8^+$ T cells. Responses to WT1 and PR1 but not BCR-ABL were detected in 10/18 healthy donors. Responses to WT1, PR1 or BCR-ABL were observed in 9/14 CML patients pre-SCT and 5/6 post SCT, often to multiple epitopes. Responses were higher in CML patients compared to healthy donors and highest after SCT. These antigen specific $CD8^+$ T cells comprised central memory ($CD45RO^+CD27^+CD57^-$) and effector memory ($CD45RO^-CD27^-CD57^+$) T cells. In conclusion, leukemia-reactive $CD8^+$ T cells derive from memory T cells and occur at low frequencies in healthy individuals and at higher frequencies in CML patients. The increased response in patients post-SCT suggests a quantitative explanation for the greater effect of allogeneic SCT.

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INTRODUCTION

Recent studies have identified a variety of antigens which elicit $CD8^+$ T cell responses against myeloid leukemias. They include minor histocompatibility antigens such as HA-1 and HA-2 [1], overexpressed self proteins such as proteinase-3 [2; 3] and Wilms tumor (WT)[4], and neoantigens created by chromosomal translocations such as BCR-ABL [5-8]. All of these antigens have been implicated in a curative graft-versus-leukemia effect of allogeneic stem cell transplantation (SCT) for CML [4; 9-11]. This evidence is based upon the occurrence of leukemia antigen-specific CTL expansions exceeding 10% of circulating T cells and coinciding with the onset of durable molecular remissions of CML [10]. $CD8^+$ T cells recognizing PR1, WT1 and BCR-ABL have also been found in patients with myeloid leukemias [8; 12] and can be elicited from healthy individuals by multiple stimulations with peptide [1; 2; 4; 8].

The observation that T cells specific for non-polymorphic leukemia antigens such as PR1, WT1 and BCR-ABL are involved in the GVL response, but are not capable of eradicating CML in the autologous setting appears at first sight contradictory. The presumed greater efficiency of alloreactive T cells to these antigens is poorly understood; it could relate to either a qualitative or a quantitative property of the cells themselves, or be due to a synergistic function with the allogeneic stimuli. In addition, the finding of autoreactive T cells in CML patients raises questions about the origin of autoreactivity to PR1, WT1 and BCR-ABL in healthy individuals. Of the three, BCR-ABL is a neoantigen, arising only after the formation of a Philadelphia chromosome positive clone. However the observation that very low frequencies of BCR-ABL positivity occur in

healthy individuals raises the possibility that healthy individuals may already have an immune response to BCR-ABL [13; 14]. In order to explore further the nature of T cells specific for leukemia antigens, we studied peripheral blood from a series of healthy individuals and patients with CML before and after SCT. The investigation of very low frequencies circulating T cells required sensitive techniques to permit the direct examination of circulating lymphocytes before their activation status is modified by in vitro expansion. For this purpose we developed a sensitive qPCR technique capable of detecting antigen-specific T cells at frequencies in the order of 1/100,000. Here we report the widespread occurrence of low frequencies of WT1 and PR1 specific T cells in both CML patients and healthy individuals. These $CD8^+$ T cells were IFN-γ producing, antigen-experienced, central memory and effector memory T cells. The major difference in antigen-specific T cells between healthy donors and patients was in their frequency, which was lowest in healthy donors, higher in patients, and highest after SCT.

MATERIALS AND METHODS

Patients and healthy controls

All donors and patients were treated at the National Institutes of Health on protocols approved by the Institutional Review Board. After informed consent, cells from patients with CML as well as their HLA-identical healthy donors were obtained from leukapheresis products (LP) before stem cell transplant. PBMCs were obtained from the apheresis products of other unrelated HLA-A*0201^+, CMV seropositive and seronegative healthy volunteers. The cells were separated using Ficoll-Hypaque density gradient centrifugation (Organon Teknika Co., Durham, NC) and subsequently frozen in RPMI 1640 complete medium (CM), (Life Technologies, Inc., Gaithersburg, MD) supplemented with 20% heat-inactivated fetal calf serum (FCS) and 10% DMSO according to standard protocols. Before use, frozen cells were thawed, washed, and suspended in RPMI-CM + 10% pooled AB serum (Sigma Chemical, St. Louis, MO). High resolution HLA class I genotyping was performed by sequence-specific PCR using genomic DNA (HLA Laboratory, Department of Transfusion Medicine, Warren G. Magnusson Center, NIH, Bethesda, MD, USA). The presence of IgG and IgM CMV antibodies in the donors was analyzed by passive latex agglutination (CMVSCAN kit, Becton Dickinson Microbiology system, Cockeysville, MD).

Cell lines

T2 cells (American Type Culture Collection, Manassas, VA) are HLA-A*0201^+ but express very low levels of surface HLA-A2.1 unless peptide-pulsed and are unable to present endogenous antigens. These cells were maintained in RPMI-CM-10% FCS.

Peptide synthesis

Peptides used in this study were prepared by Bisosynthesis, Inc. (Lewisville, TX) to a minimum purity of 95%. The identity of each of the peptides was confirmed by mass

spectral analysis. The following peptides were tested: PR1 169-177 (VLQELNVTV), derived from the azurophilic granule protein proteinase 3 [2], BCR-ABL 922-930 (GFKQSSKAL) from the b3a2 junctional region [8], WT1 126-134 (RMFPNAPYL)[4], cytomegalovirus (CMV) peptide 495-503 (NLVPMVATV) derived from the immunodominant pp65 protein [15], HIV-1 p17 Gag 77-85 (SLYNTVATL) [16] and the synthetically modified (to enhance HLA-A2 binding) gp100 peptide (209-2M) melanoma self-Ag 209-217 (IMDQVPFSV) [17].

CD8$^+$ T cell selection

CD8$^+$ T cells were purified from PBMC of healthy donors and patients using a CD8 positive isolation kit (Dynal, Oslo, Norway). Immunomagnetic beads were detached from isolated cells by using DetachaBead (Dynal) with high purity (>98%) and viability (>99%). The purity of positively and negatively selected cells was checked by flow cytometry.

RNA extraction and cDNA synthesis

RNA isolation on test samples was performed using RNeasy mini kits (Qiagen, CA, USA). Total RNA was eluted with water and stored at -80°C. For reverse transcription of mRNA and cDNA synthesis, 1 µg total RNA was reverse transcribed and stored at -20°C until qPCR was performed.

qPCR

Gene expression was measured using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer Corp.) as described previously [18; 19]. The feasibility of this approach for the analysis of antigen-specific T cell responses both in peripheral blood lymphocytes and in tumor tissues has been previously validated [20]. Primers for CD8 and IFN-γ and TaqMan probes (Custom Oligonucleotide Factory, Foster City, CA) were designed to span exon–exon junctions to prevent amplification of genomic DNA. To create a standard curve, the cDNA was generated by reverse transcription with the same technique used for the preparation of test cDNA. IFN-γ and CD8 cDNA was amplified by PCR using the same primers designed for the RT-PCR, purified and quantified by UV spectrophotometry. The number of cDNA copies was calculated using the molecular weight of each gene amplicon. Serial dilutions of the amplified genes at known concentrations were tested by RT-PCR. Quantitative RT-PCR reactions of cDNA specimens, cDNA standards and water as negative control (NTC) were conducted in a total volume of 25 µL with TaqMan Master Mix (Perkin-Elmer), 400 nM primers and 200 nM probe. Primer sequences were as follows: IFN-γ (forward) 5'-AGCTCTGCATCGTTTTGGGTT; IFN-γ (reverse) 5'-GTTCCATTATCCGCTACATCTGAA; IFN-γ (probe) FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA; CD8 (forward) 5'-CCCTGAGCAACTCCATCATGT; CD8 (reverse) 5'-GTGGGCTTCGCTGGCA; and CD8 (probe) FAM-CAGCCACTTCGTGCCGGTCTTC-TAMRA. Thermal cycler parameters included 10 minutes at 95 °C, and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Standard curve extrapolation of copy number was performed for both

IFN-γ and CD8. Sample data was normalized by dividing the number of copies of IFN-γ transcripts by the number of copies of CD8 transcripts. All PCR assays were performed in duplicates and reported as the mean. A 2-fold difference in gene expression was found to be within the discrimination ability of the assay.

Direct PCR assay of peptide-specific $CD8^+$ T cell reactivity

To screen for peptide-specific $CD8^+$ T cells, we measured IFN-γ mRNA production by $CD8^+$ T cells stimulated with candidate peptides. Preliminary experiments to optimize assay conditions were performed with $CD8^+$ T cells obtained from HLA-A*0201$^+$, CMV seropositive donors, stimulated with HLA-A*0201 associated CMV peptide $pp65_{495\text{-}503}$ pulsed T2 cells. After isolation, 1 $x10^6$ $CD8^+$ T cells were plated per well in a 96 well flat-bottom plate with 200 µl RPMI-CM supplemented with 10% human AB serum and incubated overnight at 37°C (humidity 90%, CO_2 5%) to minimize background expression of IFN-γ mRNA due to lymphocyte manipulation. $CD8^+$ T cells were then stimulated in vitro with test peptides using a protocol adapted from previous studies [2]. In brief, T2 cells were washed in serum-free medium and incubated with individual peptides at concentrations of 0.1, 1 and 10 µM at 37 °C in 5% CO_2 for 2 hours. The peptide-pulsed T2 cells were then irradiated with 7500 cGy, washed once, and added to $CD8^+$ T cells. Control wells contained $CD8^+$ T cells in the presence of unloaded T2 cells. After 3 hours of co-culture at 37 °C in 5% CO_2, cells were harvested for RNA isolation and cDNA transcription. qPCR was performed for IFN-γ mRNA expression and normalized to copies of CD8 mRNA from the same sample. Additional negative controls included HLA A*0201-negative individuals, CMV negative HLA-A*0201$^+$ individuals and T2 cells pulsed with gp100 (209-2M) peptide. Replicate tests on aliquots of a single cryopreserved apheresis sample from 3 different donors showed a coefficient of variation of 6.9-20.8%.

Production of peptide-MHC class I tetrameric complexes

Biotin-tagged HLA-A*0201 heavy chains and β_2m were expressed as insoluble inclusion bodies in E. coli strain BL21(DE3)pLysS as described previously [21]. Inclusion bodies were released by repeated freeze/thaw cycles and purified by washing with 0.5% Triton X-100 buffer (Sigma). Soluble biotinylated peptide-MHC class I (pMHCI) monomers were produced as described previously with minor modifications [21; 22]. Briefly, HLA-A*0201 heavy chain and β_2m inclusion body preparations were denatured separately in 8M urea buffer and refolded at a 1:1 molar ratio in 2-mercaptoethylamine/cystamine redox buffer (Sigma) with added synthetic peptide (BioSynthesis Inc., TX, USA). Individual complexes were produced with the following peptides: (i) NLVPMVATV (CMV); (ii) VLQELNVTV (PR1); (iii) RMFPNAPYL (WT1); (iv) SLYNTVATL (HIV-1 Gag). After buffer exchange into 10 mM Tris pH 8.1, refolded monomers were purified by anion exchange and then biotinylated as described previously using d-biotin and BirA enzyme [23]. Excess biotin was removed by gel filtration. Biotinylated monomers were conjugated by addition of fluorochrome-labeled streptavidin (ProZyme Inc., CA, USA) at a pMHC: streptavidin molar ratio of 4:1 to produce tetrameric complexes. Once prepared, tetramers were stored in the dark at 4^{o}C.

Flow cytometry

CMV tetramer-phycoerythrin (PE) and HIV1 Gag tetramer-PE were used as positive and negative controls respectively. Sample staining was performed using 3 x 10^6 PBMCs in 50 µl of 1% FCS/PBS. Tetramers (1-2 µg per test with respect to the peptide-MHC class I component) were added for 20-30 min at 37°C. Cells were washed once in PBS, 1% FCS and then stained with a titrated panel of directly conjugated antibodies to CD4, CD19, CD14, CD16, CD57, CD8, CD27 (all PharMingen, San Diego, CA), CD45RO (Dako, CA) and CD3 (Coulter). Alexa 430, FITC, Texas-Red PE, PE, Cy5PE, Cy7PE, and APC were used as fluorophores. The lymphocytes were then washed in PBS, 0·5 mM EDTA, 1% BSA, and resuspended in 1% paraformaldahyde in PBS. Data acquisition was performed with FACSDiva Calibur (BD/Pharmingen). A minimum of 1.5 x 10^6 gated cells were acquired. Data was analyzed using FlowJo software (TreeStar, San Carlos, CA). Intracellular cytokine detection was performed as described previously [24]. In brief, positively selected $CD8^+$ T cells (1 x10^6) were incubated with T2 cells loaded without or with peptide at varying doses. After 2 hours, 10 µg Brefeldin A (Sigma) was added, and after 16 additional hours, $CD8^+$ T cells were stained using CD8 PerCP, and fix/permeablized followed by staining with IFN-γ FITC (all BD/Pharmingen, San Jose, CA).

Statistical analysis

Evidence of a specific response to stimulation, as determined by qPCR studies, consisted of detection of mRNA for IFN-γ in $CD8^+$ T cells stimulated with relevant peptide versus unloaded APC (background). A cutoff value of 2.0 for the ratio of IFN-γ mRNA (corrected for CD8 mRNA) obtained from $CD8^+$ T cells stimulated with relevant epitope to that obtained from $CD8^+$ T cells stimulated with unpulsed APC was considered to be evidence of epitope specificity. The cutoff value was derived by analyzing IFN-γ mRNA transcripts detectable in $CD8^+$ T cells from both healthy donors and CML patients stimulated with gp100 (209-2M) (irrelevant peptide) compared to background. Analysis of these $CD8^+$ T cells identified a mean ratio of 0.96 (range, 0.8–1.7) with 95% and 99% confidence intervals of 0.96 ± 0.76 and 0.96 ± 1.16, respectively, a standard error of 0.09, and a standard deviation of 0.28. The cutoff ratio (stimulation index) was estimated by adding the mean to three standard deviations, which equaled to 1.8. To minimize the possibility of falsely considering $CD8^+$ T cells immunoreactive, we accepted a 2-fold increase in stimulated-unstimulated IFN-γ transcript ratio as evidence of epitope-specific reactivity.

Wilcoxon's Sum of Ranks Test was calculated to determine whether there was a statistically significant difference in IFN-γ production in response to test peptides between CML patients and healthy controls. Statistical significance was achieved when $p < 0.05$.

RESULTS.

Identification of leukemia peptide-specific CD8$^+$ T cells in healthy individuals and patients with CML

In order to determine whether PR1, WT1 and BCR-ABL peptide-specific CD8$^+$ T cells exist in healthy donors and CML patients, we looked for IFN-γ mRNA production by qRT-PCR in antigen-stimulated CD8$^+$ T cells from eighteen HLA-A*0201$^+$ healthy donors and 14 patients with CML. As controls, CD8$^+$ T cells were also stimulated with CMV pp65 (positive control) and gp100 (209-2M) (negative control) peptides. A positive response required a threshold of ≥100 IFN-γ mRNA copies/10^4 CD8 copies and a stimulation index (SI) of ≥2, where SI = IFN-γ mRNA copies/10^4 CD8 copies in peptide-pulsed T2 cell cultures / unpulsed cultures.

Responses to leukemia antigens were detected in ten of eighteen healthy individuals; the range of IFN-γ mRNA copies/10^4 CD8 copies was 145-3688 (SI of 2-1298). Five responded to stimulation with PR1 (donors 1,4, 5, 17 and 18), three to WT1 (donors 7, 8 and 9) and two to both WT1 and PR1 (donors 2 and 3). None responded to BCR-ABL or to gp100 (209-2M) peptide (Table 1, Fig. 1).

Clinical data for the patients with CML are shown in Table 2. In 9 of 14 patients pre-SCT, a CD8$^+$ T cell response to WT1, PR1 or BCR-ABL was observed, with a range of IFN-γ mRNA copies/10^4 CD8 copies of 171-6337 (SI of 2-615) (Table 1, Fig. 1). Four patients had a positive response to stimulation with both WT1 and PR1 peptides (UPNs 17, 38, 41, 199). Two responded to stimulation with all 3 peptides (UPNs 210 and 254). A CMV seronegative patient had a positive response to stimulation with WT1, PR1 and BCR-ABL but not to CMV (UPN 210). One patient had a response to PR1 and BCR-ABL (UPN 283) and two patients responded to PR1 or WT1 (UPNs 25 and 181). Six patients were tested 3-36 months after SCT, all of whom had achieved 100% donor T cell chimerism at analysis. Five patients had a response to stimulation with one or more peptides. One patient responded to both PR1 and WT1 (UPN 210), one to WT1 and BCR-ABL (UPN 327 at day 100), two to BCR-ABL alone (UPN 223 and UPN 327 when re-tested at day 360) and two to PR1, WT1 and BCR-ABL (UPNs 181 and 319). Unfortunately not enough material was available to test all 3 peptide doses in the patient who did not mount a response (UPN 283). In the case of patients UPN 181, 283, 319 & 327 samples pre and post SCT and from their respective sibling donors (healthy donors 6, 16, 2 &18) were available for analysis. For patient 223 material was available post SCT and from his donor (donor 17). Patient 319 had no response to stimulation with PR1, WT1 and BCR-ABL prior to his allogeneic SCT. His sibling donor showed substantial CD8$^+$ T cell reactivity to stimulation with PR1 and to a lesser extent with WT1 but not BCR-ABL (Table 1, Fig. 4). Post SCT, the patient had significant responses to stimulation with PR1, WT1 and BCR-ABL, suggesting the transfer of PR1 and WT1 specific CD8$^+$ T cells from donor to recipient. Patient 283 was CMV seropositive pre-SCT with a CMV seronegative donor. Post SCT he lost the ability to mount a CD8$^+$ T cell response to stimulation with the CMV peptide. Donors 17 and 18 had activity to stimulation with PR1 although post SCT no PR1 activity could be detected in their

respective recipients. Patient 327 was tested at 2 different time points, 100 and 360 days post transplant. At day 100 he had a significant $CD8^+$ response to stimulation with high dose of WT1 and smaller BCR-ABL responses. By day 360 the WT1 response had disappeared whereas the BCR-ABL response had expanded. These data suggest that pre-existing leukemia-specific donor $CD8^+$ T cells can expand in the recipient post-SCT, although formal clonotype analysis would be required for conclusive demonstration of this phenomenon; however, leukemia-specific $CD8^+$ T cell responses that were not detectable in the donor can also develop de novo in the recipient.

In individuals with leukemia the response to stimulation with PR1 and WT1 peptides was increased compared to healthy donors ($p < 0.01$). The highest amplitude responses occurred after SCT (390-38437 IFN-γ mRNA copies/10^4 CD8 copies, SI 2.0-9645). Control HLA-A*0201 negative, CMV seropositive and HLA-A*0201^+, CMV seronegative samples stimulated with $pp65_{495-503}$ and gp100 (209-2M)-pulsed T2 cells were used in each assay run. These controls were consistently negative for IFN-γ production (data not shown).

To further ascertain that the leukemia antigen response was indeed specific to the disease we studied 5 patients with solid tumors and 6 patients with other hematopoietic tumors (Table 1). None of the 5 patients with solid tumors mounted a $CD8^+$ T cell response to PR1, WT1 or BCR-ABL. One patient with secondary AML mounted a response to all doses of PR1 and WT1 peptides tested, but not to BCR-ABL (Table 1). Therefore we were able to ascertain that the antigen response is indeed appropriate for the presence of the tumor antigen. Given that >50% of healthy donors have responses to PR1 and/or WT1, we would expect a similar proportion of individuals with illnesses unrelated to CML (or AML), such as those with solid tumors, to respond in a similar way but in our series this was not observed and may be explained by suboptimal sample size.

To validate the assumption that IFN-γ mRNA transcription reflects the frequency of peptide-specific $CD8^+$ T cells and IFN-γ protein secretion, samples assayed for CMV $pp65_{495-503}$ response by qRT-PCR were compared with flow cytometric quantification of $CD8^+$ T cells specific for the peptide by tetramer or intracellular IFN-γ (IC-IFN-γ) production. In six subjects assayed for IC-IFN-γ and seven for CMV tetramer, there was a strong correlation of IFN-γ mRNA copies with IC-IFN-γ and tetramer positive T cells (R^2 = 0.92 and 0.78 respectively) (data not shown). To define linearity and limit of detection of the qRT-PCR assay, $CD8^+$ T cells from CMV seropositive individuals were stimulated with peptide-loaded T2 cells and diluted into autologous unstimulated $CD8^+$ cells. The tetramer technique was used to calibrate the number of CMV-specific T cells in the dilution. In this way the lower limit of detection by qRT-PCR was found to be one CMV-specific $CD8^+$ T cell /100,000 $CD8^+$ T cells, equivalent to approximately 100 IFN-γ mRNA copies. The same dilutions assayed by tetramer showed a lower limit of detection of 1/10,000 (Fig. 2).

$CD8^+$ T cell response to different peptide concentrations as a measure of functional avidity

To determine functional avidity, the response of $CD8^+$ T cells to stimulation with 3 concentrations of peptide (0.1, 1, 10 μM) was measured by qRT-PCR. High and low avidity T cells have been previously shown to possess different requirements for both peptide/MHC density and CD8 interaction [25; 26]. In our experiments, high avidity $CD8^+$ T cells were defined as those capable of producing IFN-γ in response to a lower concentration of peptide (0.1 μM) while intermediate and low avidity $CD8^+$ T cells were those that produced IFN-γ in response to a higher concentration of peptide (1 and 10 μM respectively). To examine the issue of functional avidity, we determined the ratio of high to low avidity $CD8^+$ T cell responses in healthy donors and CML patients by measuring the number of IFN-γ mRNA copies/10^4 CD8 copies produced by cells stimulated with low or higher peptide concentrations respectively (Fig. 3). We observed that in CML patients, low avidity $CD8^+$ T cell responses to PR1, WT1 and BCR-ABL were more abundant than high avidity responses (median high/low avidity ratios of 0.73, 0.32 and 0.04 respectively). In contrast, in healthy donors, $CD8^+$ T cell responses to PR1 and WT1 were mostly high avidity (median ratios of 214 and 2.2 respectively). The difference in response in the two groups was statistically significant ($p = 0.01$ and $p < 0.05$ respectively). In contrast high and low avidity CMV specific $CD8^+$ T cell responses were equally represented and were not statistically significantly different in the two groups (p=0.12)

Phenotypic analysis of CMV, PR1 and WT1 specific $CD8^+$ T cells

Antigen-specific $CD8^+$ T cells selected by HLA-A*0201 tetramers were analyzed for expression of CD45RO, CD27 and CD57 for characterization of naïve, memory and effector phenotype. In two healthy donors, the frequencies of PR1 and WT1-tetramer positive cells were high enough to permit phenotypic characterization (Figure 3). Due to technical difficulties with the synthesis and quality of the HLA-A*0201 BCR-ABL tetramer we were unable to study the phenotype of the BCR-ABL specific $CD8^+$ T cells. CMV-tetramer specific $CD8^+$ T cells had a predominant phenotype of effector memory cells and a minor population of central memory T cells (3a). In contrast, in healthy donors and CML patients PR1 and WT1 tetramer-specific $CD8^+$ T cells displayed two phenotypes: central memory ($CD45RO^+CD27^+CD57^-$) and terminally differentiated effector memory ($CD45RO^-CD27^-CD57^+$) (Figure 3 and Table 4) with a predominant phenotype of central memory. After stem cell transplantation, a shift towards more effector memory phenotype was noted, probably implying an ongoing GVL effect.

DISCUSSION

This study provides direct evidence that memory $CD8^+$ T cell responses recognizing several leukemia-associated self-antigens exist at low frequencies in healthy individuals and to a greater extent in patients with CML. Using qRT-PCR, we found that $CD8^+$ T-cells recognizing leukemia-associated antigens WT1 and PR1 are present in low frequencies in more than 50% of healthy individuals. In contrast, none had a response to

the BCR-ABL junctional peptide. The presence of such $CD8^+$ T cells in healthy subjects confirms WT1 and PR1 to be self-antigens and concords with the ability to elicit PR1- and WT1-specific cytotoxic T lymphocytes (CTLs) from healthy individuals by repeated antigen stimulation [3; 4], the finding of antibodies to WT1 in up to 16% of healthy subjects [27; 28], and the occurrence of proteinase-3 specific antibody and T cell responses in most healthy individuals and in patients with Wegener's granulomatosis [29-31]. Indeed, natural antibodies reacting with a variety of self-antigens have been detected in serum of healthy individuals [32]. Nevertheless the frequency of WT1 and PR1 specific $CD8^+$ T cells in our study was extremely low and (with two exceptions) below the limit of detection using tetramers. Thus WT1 and PR1 appear to behave as tissue- but not leukemia-specific antigens in the same category as antigens identified in solid tumors [33]. The absence of a response to BCR-ABL suggested that at least in HLA-A*0201 positive individuals this particular BCR-ABL sequence was not antigenic. While this could indicate that BCR-ABL is not antigenic because it is a true neoantigen absent from the normal antigenic environment, we cannot exclude the possibility that antigenic BCR-ABL sequences exist in the peripheral T cell repertoire in individuals of other HLA types.

While the frequencies of responses to CMV antigen were comparable in healthy subjects and patients, we found that $CD8^+$ T cells to WT1, PR1 and BCR-ABL circulate in patients with CML at significantly higher frequencies. Even higher frequencies to some of these antigens (>0.5%) were found after allogeneic SCT, suggesting that these leukemia antigen-associated $CD8^+$ T cells expand in the recipient post transplant and contribute to remission. Responses to BCR-ABL occurred in 3/9 pre- and 5/6 patients post-SCT. In all, 7/14 patients pre-SCT and 4/6 post-SCT had responses to multiple tumor antigens. In contrast with previous reports [10] PR1-specific $CD8^+$ T cells were detectable even in patients not treated with alpha-interferon. This may be explained by the higher sensitivity of the qRT-PCR assay compared with tetramer staining.

The qRT-PCR IFN-γ mRNA assay allowed for the identification of very low frequencies of circulating antigen-specific $CD8^+$ T cells following brief in vitro exposure to candidate peptides. This assay showed a linear relationship between IFN-γ mRNA copy number and peptide-specific $CD8^+$ T cell frequencies. Using a CMV pp65 peptide, the IFN-γ copy number detected by qRT-PCR correlated well with IFN-γ protein production by IC staining (R^2 = 0.98) and with antigen-specific $CD8^+$ T cells measured by tetramer staining (R^2 = 0.78). Furthermore, we demonstrated that qRT-PCR was at least 10 times more sensitive in the detection of CMV-specific T cells than tetramer staining. qRT-PCR has the advantage over other techniques of being faster, more sensitive, and requiring fewer cells. Moreover, since cell expansion is not required to detect T cell reactivity, the method allows the detection of functional antigen-specific T cells unmodified in frequency or functional state by *in vitro* expansion.

In addition to quantitative differences in antigen-specific T cells between healthy subjects and CML patients we sought qualitative differences in T cell responses between patients and healthy donors. Using three peptide concentrations, we characterized functional avidity by measuring the ability of $CD8^+$ T cell populations to respond to the stimulation provided by three logarithmically different peptide concentrations [34]. We detected both

high- and low-avidity antigen-specific $CD8^+$ T cells in healthy donors and CML patients irrespective of their transplant status. Patients showed an increase in low avidity $CD8^+$ T cell responses in both autologous and allogeneic settings compared to healthy donors and the difference was statistically significant. These results suggest that the repertoire of leukemia antigen specific $CD8^+$ T cells is diverse both in terms of clonal composition and efficiency of peptide recognition. This observation is further supported by a recent paper by Molldrem et al [35] who showed that distinct high or low avidity PR1 CTLs can be expanded from the peripheral circulation of healthy individuals. However, only low-avidity CTLs were detected in the peripheral blood of patients with CML and it was not possible to elicit high-avidity PR1-CTLs from untreated CML patients. The tendency for a preponderance of low avidity $CD8^+$ T cells in CML patients may be explained by the loss of high-avidity $CD8^+$ T cells by apoptosis following exposure to supra-optimal antigen density on leukemic antigen-presenting cells (APC). When a malignant cell overexpressing tumor-associated self-antigens expands, high-avidity T cells with specificity for those tumor antigens might be selectively eliminated over time through clonal deletion. This is similar to the process of clonal exhaustion of high-avidity $CD8^+$ T cells that occurs during viral infection [36], also shown for $CD4^+$ cells [37].

The finding of low frequencies of IFN-γ producing WT1- and PR1-specific $CD8^+$ T cells in healthy individuals suggested that they belonged to an antigen-experienced memory cell population since naïve T cells characteristically produce cytokines other than IFN-γ [38]. To better characterize the functional status of these cells in patients and healthy individuals, we used multiparametric flow cytometry to study the surface phenotype of PR1 and WT1-tetramer specific $CD8^+$ T cells. Two healthy donors had sufficiently high frequencies of PR1- and WT1-specific $CD8^+$ T cells to allow the study of their functional phenotype. Overlapping markers such as CD45RA, CD45RO, CD28, CD27, CD57 and CCR7 have been used to identify the differentiation state of antigen-specific $CD8^+$ T cells [38 39-41]. Based on CD45RO, CD27 and CD28 expression and analysis of the replicative history and clonality of the T-cell populations [42], phenotypically distinct and sequential stages of $CD8^+$ T-cell differentiation have been proposed [43]. We found that, in both patients and healthy subjects, leukemia-reactive $CD8^+$ T cells displayed a similar mixed $CD45RO^{dim}/CD27^-/CD57^+$ and $CD45RO^{bright}/CD27^+/CD57^-$ phenotype corresponding to terminally differentiated effector/memory and central memory phenotypes respectively. Following allogeneic transplantation, there was a shift towards more effector phenotype implying an ongoing graft versus leukemia effect.

Taken together, our data confirm the presence of circulating $CD8^+$ T cells recognizing several leukemia-associated antigens both in CML patients pre- and post-transplant and in healthy subjects. The finding of $CD8^+$ T cells specific for up to three antigens in CML patients suggests that the leukemia presents a number of epitopes that could render it susceptible to T cell attack for example in GVL responses. IFN-γ production and surface phenotype confirmed leukemia antigen-reactive $CD8^+$ T cells to be memory cells distributed between the central memory and effector memory pools. The presence of terminally differentiated effectors suggests a persisting antigenic stimulus, even in healthy individuals, as also occurs in chronic infections with CMV, EBV and HIV [44]. While there were significant differences in the functional avidity of $CD8^+$ T cell

responses between patients and healthy donors, and a tendency towards more effector $CD8^+$ T cells following allogeneic transplantation, the major difference was the much higher frequency of leukemia antigen-specific $CD8^+$ T cells in CML patients and the occurrence of BCR-ABL-specific $CD8^+$ T cells only in these individuals. It is thus possible that BCR-ABL represents a true leukemia neoantigen absent from the antigenic environment of healthy individuals. In this study differences in antigen-specific $CD8^+$ T cell frequencies were the only explanation reconciling the apparent failure of an antileukemic effect in non-transplanted CML patients, with the presumed success of functionally similar but higher frequency T cells exerting a GVL effect post transplant. The implication of this finding is that increasing the T cell frequency, for example by peptide vaccination, may be an important goal when using immunotherapy as treatment for CML. Furthermore it should be feasible to boost existing memory T cell responses to leukemia antigens both in patients and in healthy stem cell donors.

LEGENDS AND FIGURES

Figure 1. $CD8^+$ T cell response to stimulation with the HLA-A*0201-restricted peptides CMV pp65 (a), PR1 (b), WT1 (c) and BCR-ABL (d) in 18 healthy donors and 14 CML patients. $CD8^+$ selected T cells were incubated for 3 hours with unpulsed APC, or APC pulsed with 3 doses of peptide (0.1, 1 and 10 μM). Values represent copies of IFN-γ mRNA per 10^4 copies of CD8 mRNA. Because of limitation in the amount of PBMC available, the intermediate dose testing was omitted in certain cases and the data on intermediate and low avidity are presented together. The $CD8^+$ T cell response to stimulation with each particular peptide was calculated by subtraction of IFN-γ mRNA copies/10^4 CD8 copies induced by unpulsed APC (background) from that induced by peptide-pulsed APC. Values greater than 100 IFN-γ mRNA copies per 10^4 copies of CD8 and at least two times that of background were defined as positive responses. Bars represent the median number of IFN-γ mRNA copies/10^4 CD8 copies for each condition. Responses to stimulation with PR1 and WT1 were significantly higher in the CML group. Responses to stimulation with BCR-ABL were only detectable in the CML group.
a. CMV response
b. PR1 response
c. WT1 response
d. BCR-ABL response

Figure 2. 10^6 $CD8^+$ T cells were stimulated with pp65 CMV peptide-pulsed T2 cells for three hours and then diluted logwise into unstimulated, autologous $CD8^+$ T cells. The number of CMV specific $CD8^+$ T cells in the starting material determined by tetramer assay was used to calibrate CMV specific $CD8^+$ T cells in each dilution and correlate this with the number of IFN-γ mRNA copies such that the lowest concentration contained 1 CMV-positive $CD8^+$ T cell/10^6 non-stimulated $CD8^+$ T cells. RNA was then extracted for qRT-PCR. The lower limit of detection by the tetramer and PCR assay was 1/ 10,000 and 1/100,000 CMV specific $CD8^+$ T cells respectively.

Figure 3. High and low avidity $CD8^+$ T cell responses determined by sensitivity to peptide concentration in healthy donors and CML patients. Stimulating $CD8^+$ T cells with 0.1 μM and 10 μM of CMV pp65 (square), PR1 (circle), WT1 (triangle) and BCR-ABL (diamond) determined high and low avidity responses, respectively. Results shown are the ratios of high to low avidity $CD8^+$ T cell responses, calculated for individual healthy donors (filled symbols) and CML patients (open symbols). Ratios were obtained by the following calculation: number of IFN-γ mRNA copies/10^4 CD8 copies with 0.1 μM peptide/ number of IFN-γ mRNA copies/10^4 CD8 copies with 10 μM peptide. Bars represent the median high/low avidity ratio for each condition. $CD8^+$ T cell responses to PR1, WT1 and BCR-ABL in CML patients were mostly low avidity, whereas $CD8^+$ T cell responses in healthy donors were skewed towards high avidity responses ($p = 0.01$ and $p < 0.05$ respectively). CMV responses were not statistically different in the 2 groups ($p = 0.12$).

Figure 4. Phenotypic characterization of tetramer-positive $CD3^+CD8^+$ T cells. Analysis of PBMCs was performed by 6-color flow cytometry in CML patients pre- and post SCT and 2 healthy donors whose $CD8^+$ T cell frequencies to PR1 and WT1 were high enough to be visualized by tetramer staining. CD45RO, CD27 and CD57 phenotype of CD3/CD8-gated tetramer positive lymphocytes was analyzed. The majority of CMV tetramer-$CD8^+$ T cells were $CD45RO^-CD27^-CD57^+$ (blue) consistent with an effector memory phenotype (4a). PR1 and WT1 tetramer positive $CD3^+CD8^+$ T cells in two CML patients pre-SCT and two patients post-allogeneic SCT showed a mixture of $CD45RO^+CD27^+CD57^-$ (red) and $CD45RO^-CD27^-CD57^+$ (blue) with the majority of the tetramer gated cells showing the former phenotype (b&c). The same held true when PR1- and WT1 tetramer positive T cells in two healthy donors were studied. Representative data are presented here.

a. CMV positive control (UPN 283)
b. CML patient pre-SCT PR1 (UPN 210)
c. CML patient post SCT PR1 (UPN 319)
d. CML patient post SCT WT1 (UPN 319)
e. Healthy donor (12) WT1
d. Healthy donor (2) PR1

Table 1. Reactivity of $CD8^+$ T cells from healthy donors, CML patients pre- and post SCT and patients with other hematopoietic or non-hematopoietic tumors to test peptides. Data are represented as stimulation index that corrects the amount of IFN-γ mRNA copy number expressed by $CD8^+$ T cells exposed to relevant peptide over constitutive expression of same cytokine (exposure to unloaded APC) as measured by the direct quantitative, RT-PCR assay. *- Stimulation indices scored as (-) represent reactivities <2 over background. The – scores for CMV stimulation were only seen in CMV seronegative individuals. Note: due to limited amount of material not all peptides and concentrations were tested in every case. Patient 327 was tested at two time points post SCT, at days 100 and 365. RCC: renal cell carcinoma, RAEBT: refractory anemia with excess blasts in transformation, RA: refractory anemia, MDS: myelodysplastic syndrome, CMML: chronic myelomonocytic leukemia.

A.

	CMV (μM)			PR1 (μM)			WT1 (μM)			BCR-ABL (μM)		
Healthy donors	0.1	1	10	0.1	1	10	0.1	1	10	0.1	1	10
1	- *	-	-	1298		-	-	-	-	-		-
2	62		75	29	17	6	2	2	-	-	-	-
3	-	-	-	13	2	-		5	6	-	-	-
4	-			2	-	-	-	-	-	-		-
5	-			-		2	-		-	-		-
6	24		18	-	-	-	-	-	-			
7	-	-	-	-	-	-	16	370	1067	-	-	-
8	23			-	-	-	-		2	-	-	-
9	-			-		-	5		2			
10	10			-	-	-	-	-	-	-	-	-
11	10			-	-	-	-	-	-	-	-	-
12	108		87	-	-	-	-	-	-	-	-	-
13	2339		5935	-	-	-	-	-	-			
14	20303		5805	-	-	-	-	-	-			
15	-	-	-	-	-	-	-	-				
16	-	-	-	-	-	-	-	-	-	-	-	-
17	-			-	-	76	-	-	-	-	-	-
18	8.2			2.3	-	-	-	-	-	-	-	-
CML pre-SCT (UPN)												
16	-			-		-	-		-	-		-
17	-			-		145	175		-			
25	-			-	-	-	-	-	152			
38	-			4	-	4	4	-	4			
41	-			3		-	-		8	-	-	-
181	-			-	-	3.2	-	-	-	-	-	-
199	-			-	323		-	237				
210	-	-	-	615	136	156	-	336	-	-	34	216
241	42	32	21	-	-	-	-	-	-	-	-	-
254	-			2		2	2		2	2		2
262	-			-		-	-		-	-		-
283	43			17	11	12	-	-	-	-	-	2
319	24	21	24	-	-	-	-	-	-	-	-	-
327	-			-		-	-		-			
CML post SCT												
181	8.5			-	2.5	-	-	2.1	-	-		2.0
210	1133		1712	9643	-	-		684	9	-	-	-
223	-			-	-	-	-	-	-	-		2.5
283	-			-		-	-		-	-		-
327 (d100)	53			-	-	-	-		136	2.5		2.5
327 (d360)	41			-	-	-	-	-	-	-	-	29
319	2	7417	9	-	3	-	3	-	-	-	3.5	-

B.

		PR1 (μM)			WT1 (μM)			BCR-ABL (μM)		
Patient	Diagnosis	0.1	1	10	0.1	1	10	0.1	1	10
154	RCC	-	-		-	-	-	-		
167	RCC	-	-	-	-		-	-	-	-
209	RCC	-	-	-	-	-	-	-	-	-
214	RCC	-	-	-	-	-	-	-	-	-
215	Sarcoma	-	-	-	-	-	-	-	-	-
52	RAEBT	-	-	-	-	-	-	-	-	-
187	CMML				-		-			
227	MDS				-		-			
350	RAEBT	29.9	8.8	6.3	11	5.6	4.3	-	-	-
PT 3	MDS	-			-	-	-			
PT 4	RA				-	-	-			

Table 2. Characteristics of 14 CML patients pre-SCT (a) and 6 CML patients post SCT (b). Abbreviations: CML-CP: CML chronic phase, CML-LB: CML lymphoid blast crisis, CML-MBC: CML myeloid blast crisis, SCT: stem cell transplant, HU: hydroxyurea, IFN-α: interferon-alpha, BU: busulphan, Hyper CVAD: cyclophosphamide, doxorubicin, vincristine, and dexamethasone, ARA-C: cytosine-arabinoside, HLA id: HLA identical sibling transplant, MRD: minimal residue disease- BCR-ABL positive by RT-PCR, CR: molecular remission, N/A: not available

UPN	Disease stage at analysis	BCR-ABL translocation	Time from diagnosis to analysis (months)	Treatment	T cell response to PR1	T cell response to WT1	T cell response to bcr-abl
16	CML-CP	b3a2	2	HU	N	N	N
17	CML-CP	N/A	4	BU, HU	Y	Y	
25	CML-CP	N/A	15	BU	N	Y	
38	CML-CP	N/A	3	HU	Y	Y	
41	CML-CP	b3a2	24	HU	Y	Y	N
181	CML-CP	b3a2	2	HU, IFN-α	Y	N	N
199	CML-CP	b2a2	15	HU	Y	Y	
210	CML-LBC 2nd CP	b2a2	4	HU, IFN-α, Hyper CVAD-3 cycles	Y	Y	Y
241	CML-CP	b3a2	3	HU	N	N	N
254	CML-CP	N/A	8	HU	Y	Y	Y
262	CML-CP	b3a2/b2a2	5	HU	N	N	N
283	CML-MBC 2nd CP	b3a2/b2a2	84	HU, Gleevec	Y	N	Y
319	CML-CP	b3a2	4	HU, anagrelide	N	N	N
327	CML-CP	b3a2	9	HU, ARA-C	N	N	

B.

Post SCT	Date from SCT to analysis	Disease stage at SCT	Treatment before SCT	Type of SCT	DLI-interval to analysis	Disease Status at analysis	GVHD	T cell response to PR1	T cell response to WT1	T cell response to bcr-abl
181	36 m	CML-CP	HU	HLA-id	18 m	MRD	acute I-skin, chronic-skin limited	Y	Y	Y
210	180 d	CML 2nd CP	HU, IFN-α, Hyper CVAD-3 cycles	HLA-id	N	CR	chronic-skin limited	Y	Y	N
223	35 m	CML-CP	HU, IFN-α	HLA-id	27 m	CR	acute I-liver	N	N	Y
283	23 m	CML-MBC 2nd CP	Gleevec	HLA-id	20 m	CR	acute II-skin chronic skin-extensive	N	N	N
319	100 d	CML-CP	HU, anagrelide	HLA-id	55 d	MRD	acute I-skin, chronic skin	Y	Y	Y
327	100 d	CML-CP	HU, ARA-C	HLA-id	55 d	MRD	none	N	Y	Y

Table 3. Identification and characterization of ex-vivo CMV, PR1 and WT1 tetramer-positive $CD3^+$ $CD8^+$ T cells in healthy donors and CML patients

		% tetramer positive $CD8^+$ T cells	
UPN	Tetramer (%)	Central Memory	Effector Memory
283(Pre-SCT)	CMV- 3.2	23	77
210 (Post-SCT)	CMV- 1.81	18	82
210 (Pre-SCT)	PR1- 0.17	64	36
210 (Pre-SCT)	WT1- 0.12	70	30
283 (Pre-SCT)	PR1-0.16	64	36
319 (Post-SCT)	PR1- 0.45	56	44
319 (Post-SCT)	WT1- 0.07	59	41
210 (Post-SCT)	PR1- 0.15	58	43
Healthy donor (2)	PR1- 0.13	63	37
Healthy donor (12)	WT1- 0.056	75	25

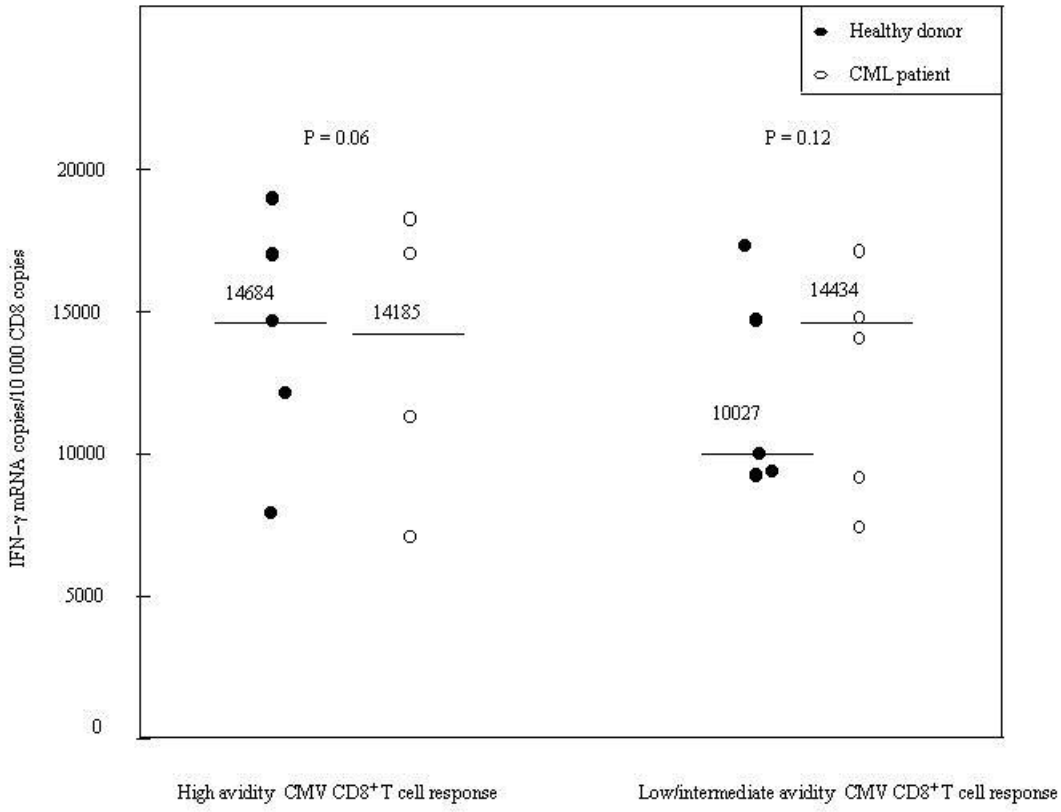


Figure 1a

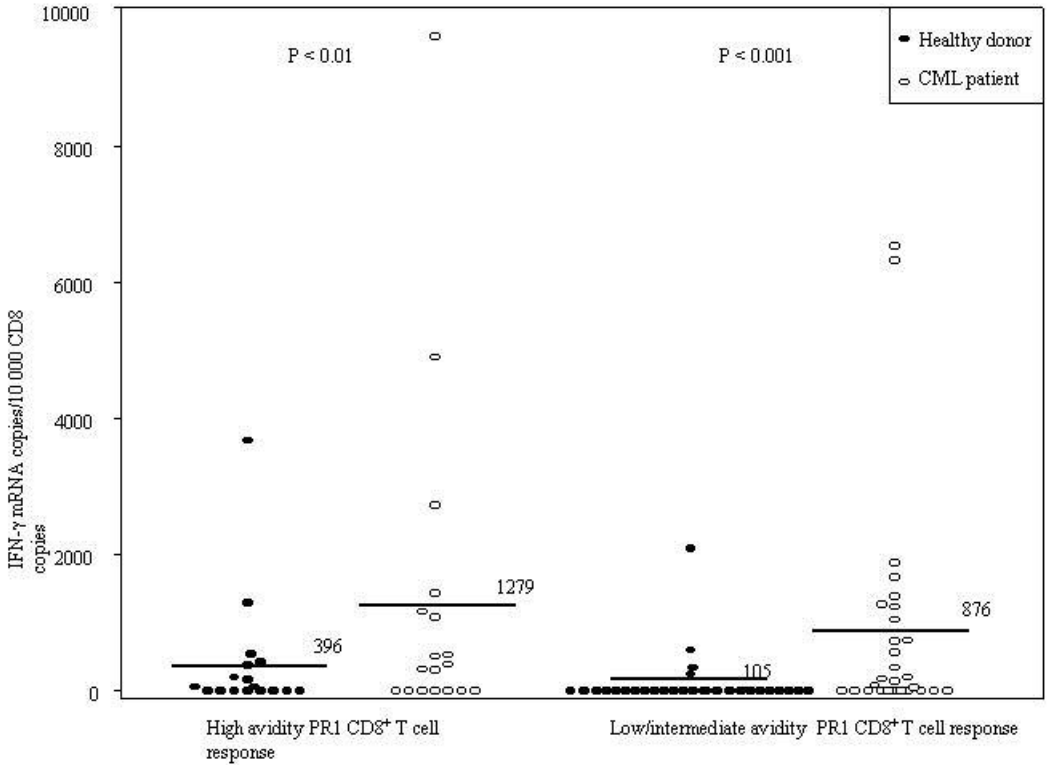


Figure 1b

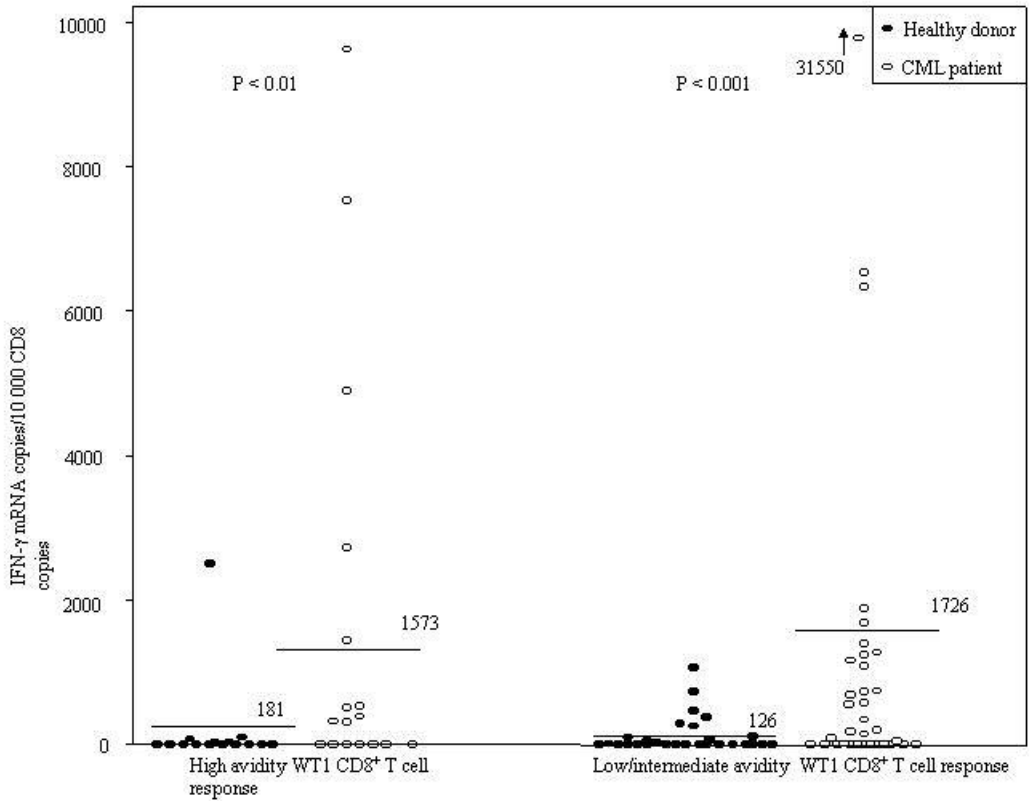


Figure 1c

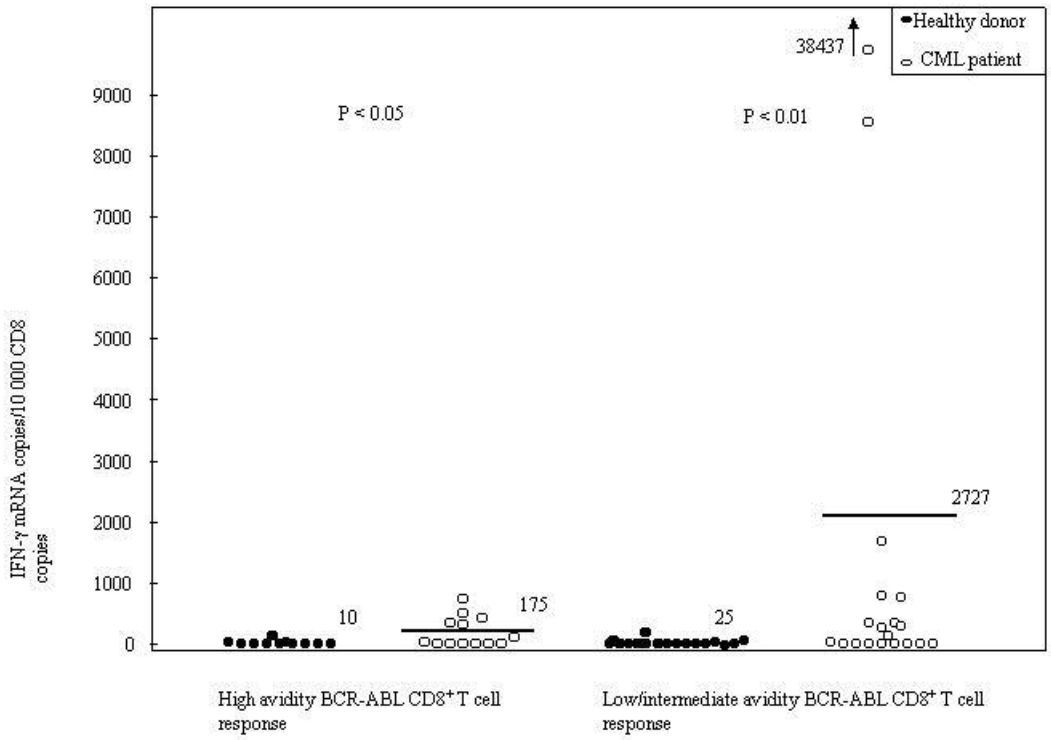


Figure 1d

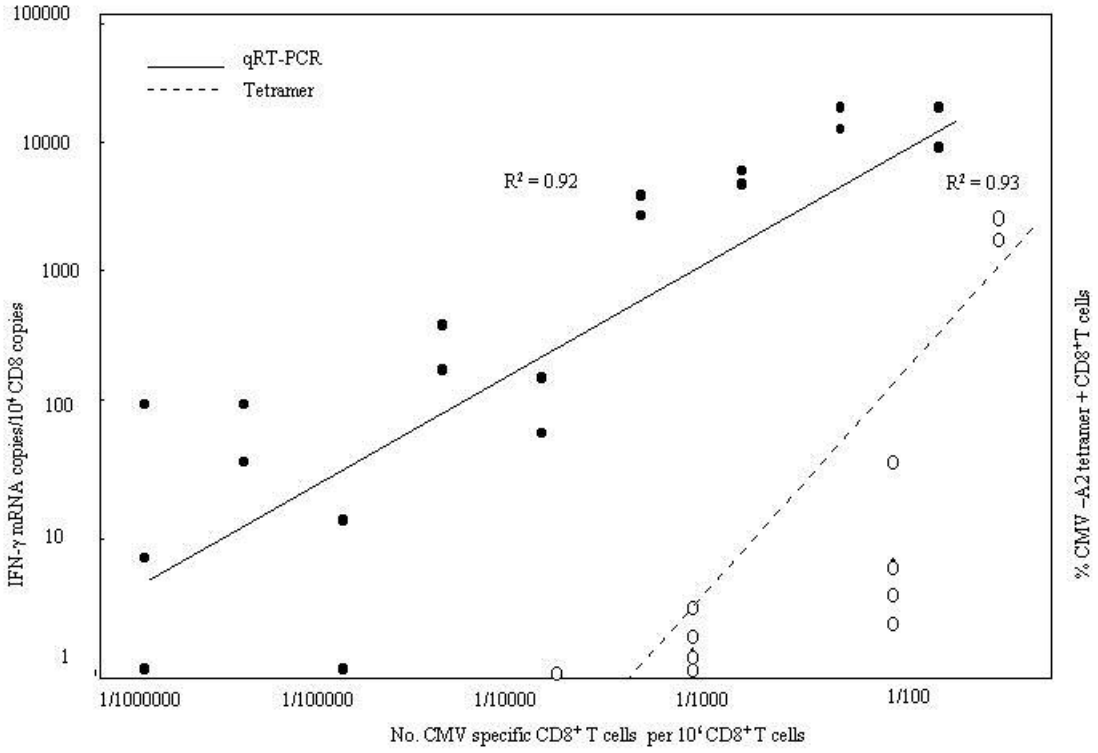


Figure 2

s

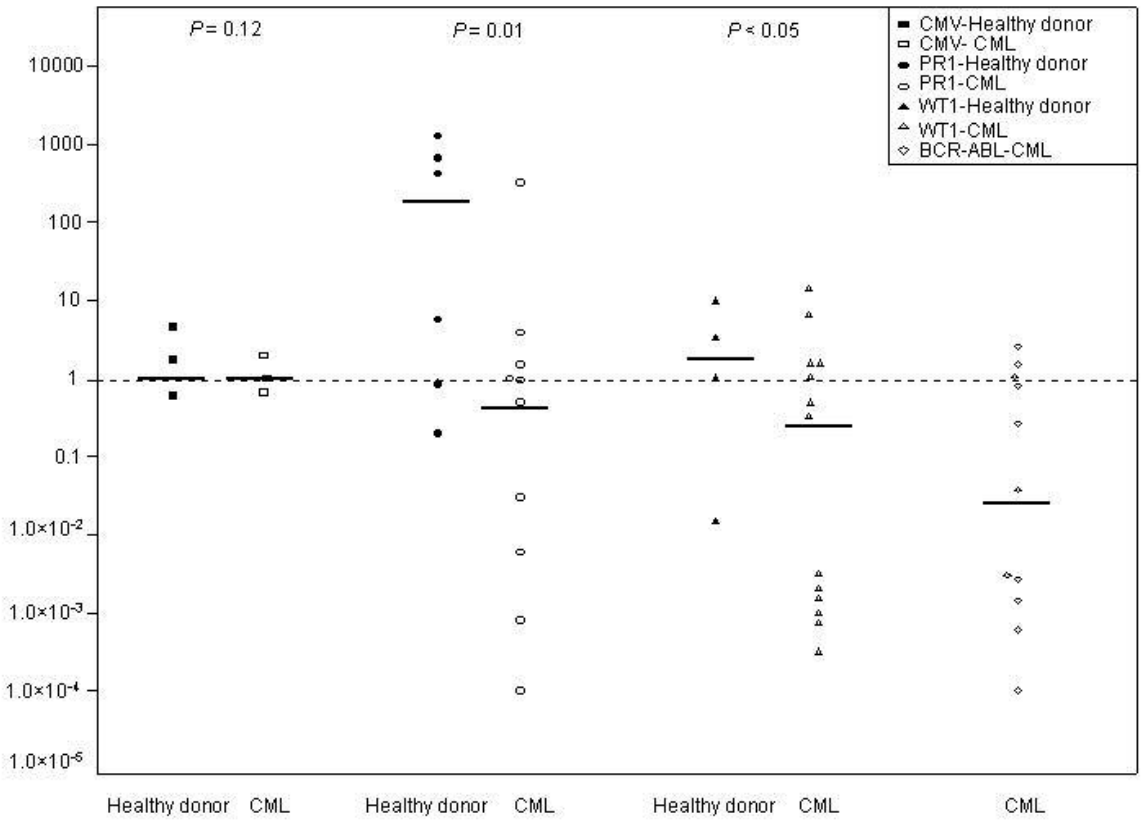


Figure 3

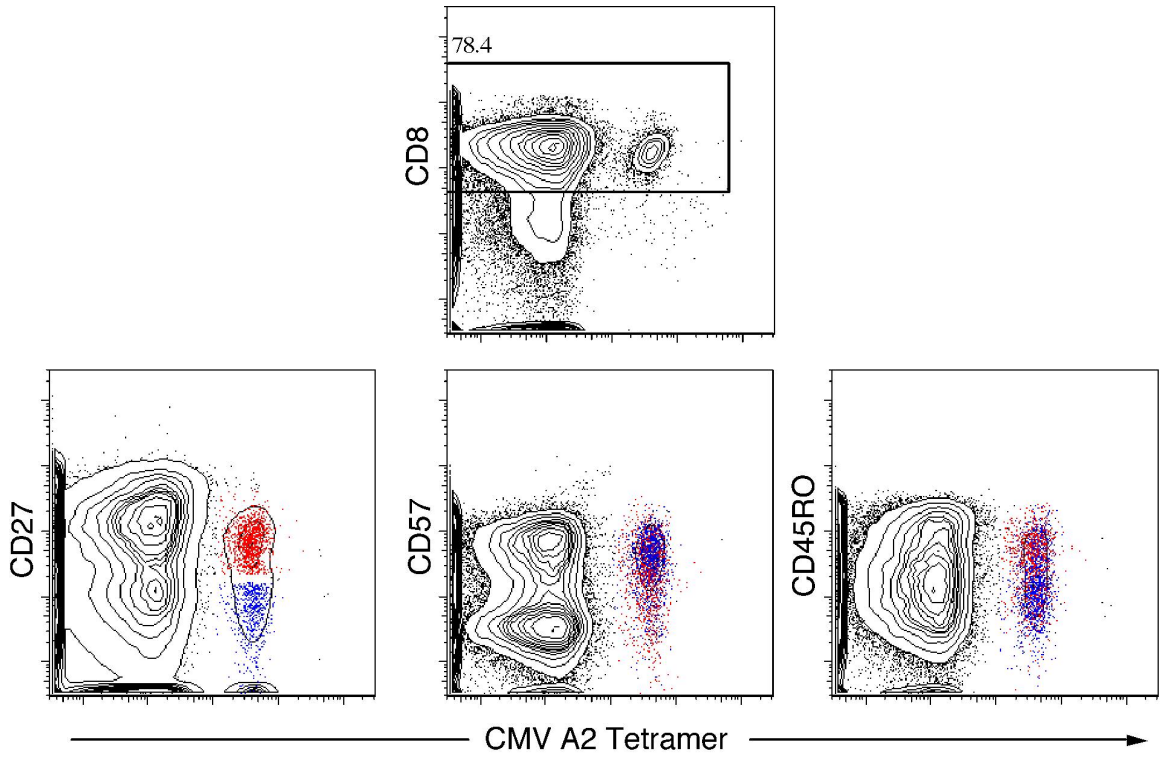


Figure 4a

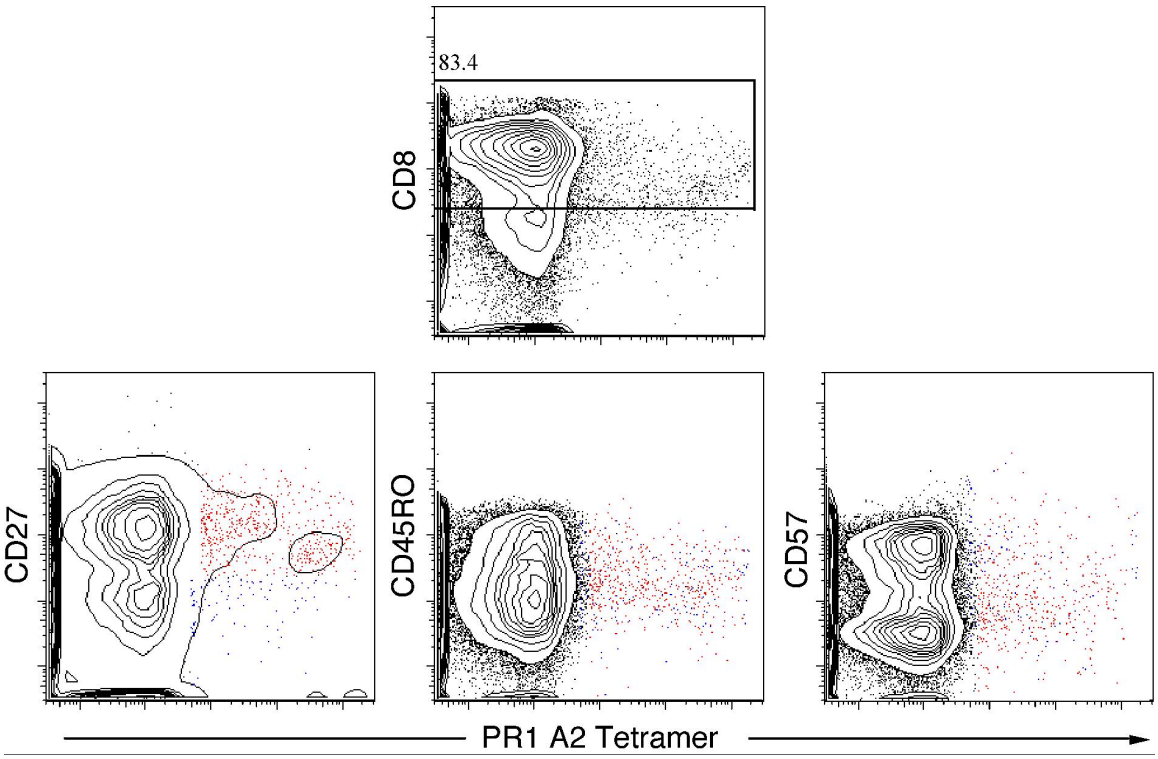


Figure 4b

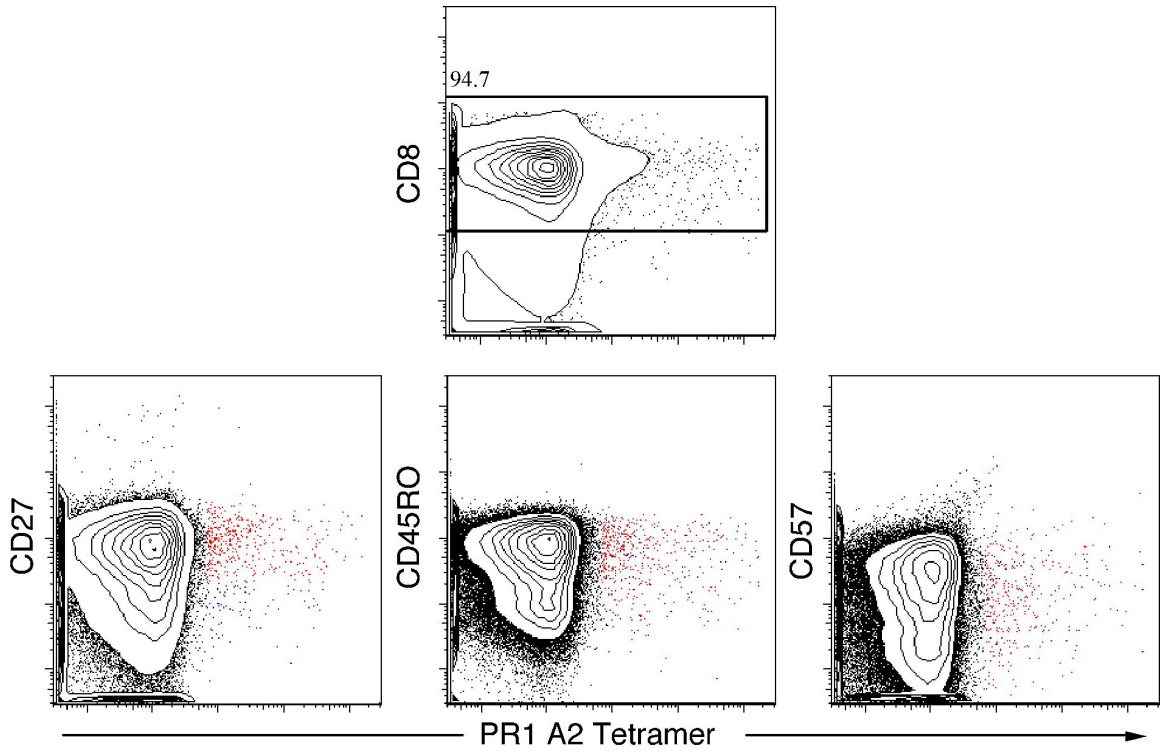
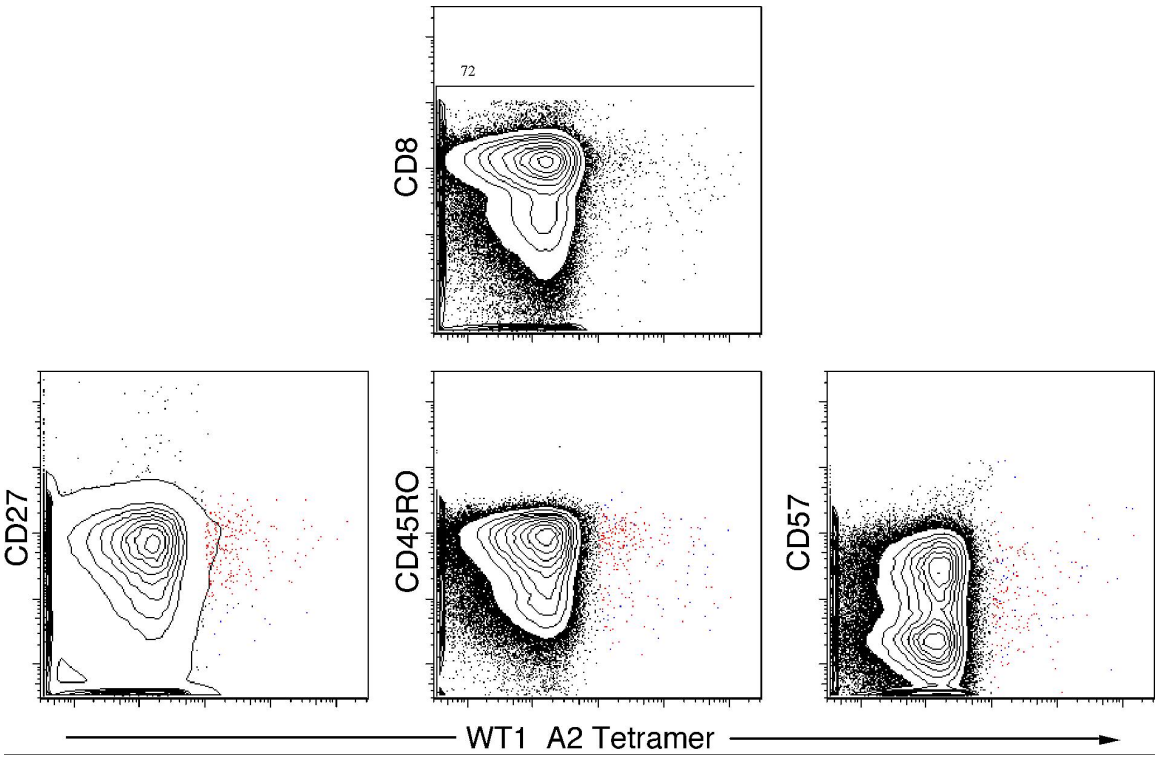


Figure 4c



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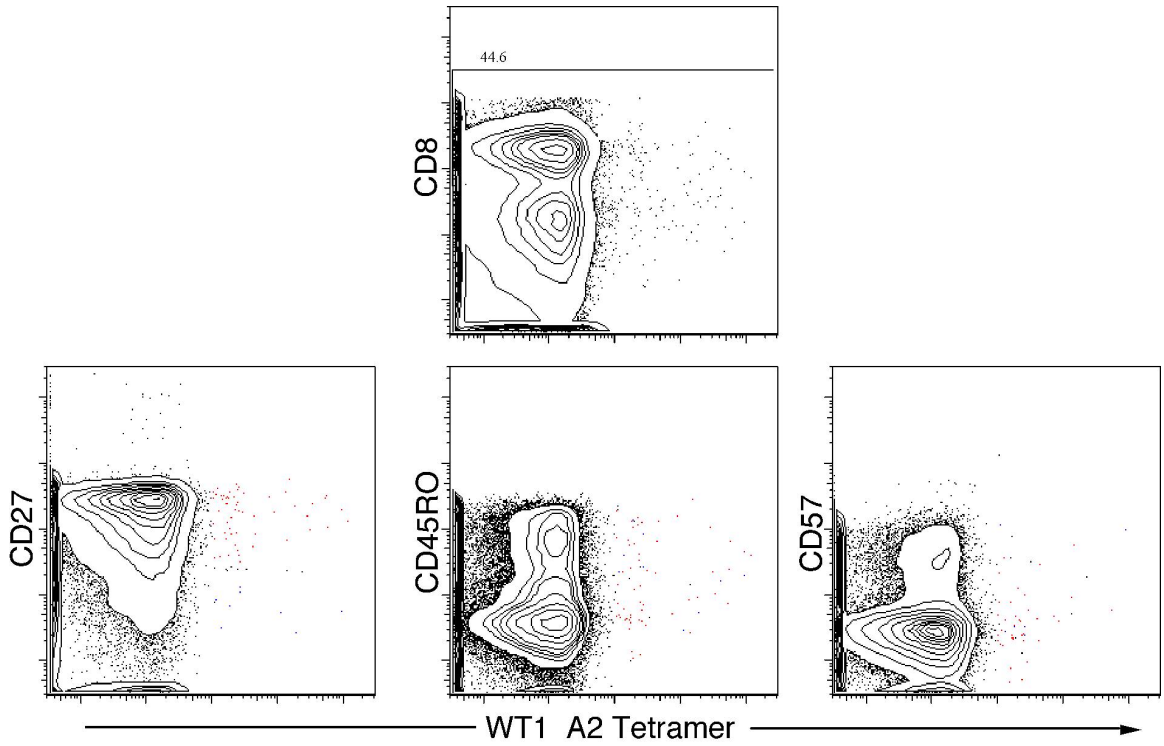

Figure 4e

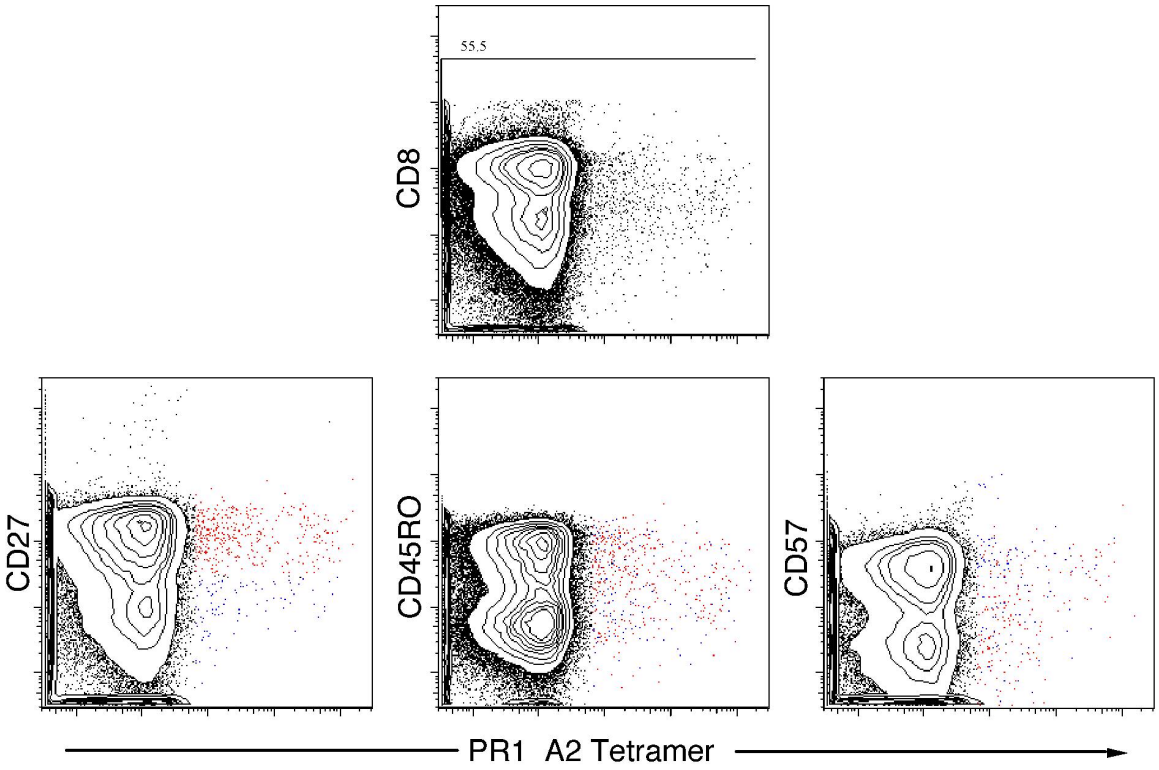

Figure 4f

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