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## **Therapeutic Targeting of the BCR Associated Protein CD79b in a TCR-based Approach Is Hampered by Aberrant Expression of CD79b**

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4 Immunotherapy of B cell malignancies using CD19-targeted chimeric antigen receptor (CAR)-transduced T cells or CD20-targeted therapeutic monoclonal antibodies has shown clinical efficacy. However, refractory disease and the emergence of antigen-loss tumor-escape variants after treatment demonstrate the need to target additional antigens. Here, we aimed to target the B cell receptor associated protein CD79b by a T cell receptor (TCR)-based approach. Since thymic selection depletes high avidity T cells recognizing CD79b-derived peptides presented in self-HLA molecules, we aimed to isolate T cells recognizing these peptides presented in allogeneic HLA. Peptide-HLA tetramers composed of CD79b peptides bound to either HLA-A2 or HLA-B7 were used to isolate T cell clones from HLA-A\*0201 and B\*0702-negative individuals. For three distinct T cell clones CD79b specificity was confirmed through CD79b gene transduction and CD79b-specific shRNA knockdown. The CD79b specific T cell clones were highly reactive against CD79b-expressing primary B cell malignancies whereas no recognition of nonhematopoietic cells was observed. Although lacking CD79b cell surface expression, intermediate reactivity towards monocytes, hematopoietic progenitor cells and T cells was observed. Quantitative RT-PCR revealed low CD79b gene expression in these cell types. Therefore, aberrant gene expression must be taken into consideration when selecting common, apparently lineage-specific self-antigens as targets for TCR-based immunotherapies.

## Introduction

Clinical studies using chimeric antigen receptor (CAR)-modified T cells or targeted monoclonal antibodies have shown the efficacy of immunotherapy to treat different hematological malignancies. The adoptive transfer of T cells modified to express CD19-targeted CARs have led to complete remissions in patients suffering from chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL)<sup>1-4</sup>. Due to its high expression on malignant cells, CD19 can be efficiently targeted by CAR-transduced T cells. Similarly, CD20-targeting therapeutic monoclonal antibodies such as Rituximab have caused complete remissions in patients treated for follicular lymphoma<sup>5, 6</sup> and diffuse large B cell lymphoma<sup>7, 8</sup> in combination with chemotherapy. Besides on malignant B cells, CD19 and CD20 are also highly expressed on healthy B cells causing depletion of the B cell compartment in patients treated with CD19-targeted or CD20-targeted immunotherapies<sup>1, 3-5, 9</sup>. However, side effects of long-term B cell depletion are manageable which indicates that B cell aplasia can be tolerated<sup>9-13</sup>.

Although successful in several patients, malignancies refractory to CD20-targeted therapy and the emergence of CD19-negative tumor-escape variants after treatment with CD19-targeting CAR-transduced T cells<sup>4</sup> demonstrate the need to identify additional targets for the treatment of B-lineage malignancies. We hypothesized that the B cell receptor (BCR) associated protein CD79b presents a suitable target for immunotherapy since CD79b membrane expression is restricted to the B-cell compartment and can be found on many B cell malignancies<sup>14-16</sup>. The BCR associated heterodimer CD79a/b is critically involved in BCR transport<sup>17</sup> and functionality by providing signaling capacity<sup>18-22</sup> to regulate processes such as allelic exclusion, proliferation, differentiation, anergy and apoptosis<sup>23-25</sup>. CD79b is encoded by gene *B29*<sup>26</sup> and through the event of alternative splicing can produce different transcripts<sup>27</sup>. The truncated variant of CD79b, missing almost entirely its extracellular domain, has been implicated to render CLL unresponsive to BCR-induced apoptosis<sup>28</sup>. Furthermore, a high percentage of malignant cells expressing cell surface CD79b in combination with other phenotype markers has been associated with shortened overall survival in B-CLL patients<sup>29, 30</sup>. An alternative strategy for the treatment of cancer is the use of T cell receptor (TCR) gene transfer therapy<sup>31, 32</sup>. T cells engineered to express TCRs with defined antigen specificity have already successfully been used in the treatment of solid tumors<sup>33</sup>. Furthermore, only very low quantities of agonistic peptide-HLA complex are required for TCR triggering and T cell activation<sup>34-36</sup> while CAR and antibody-based approaches necessitate greater abundance of the antigen<sup>37</sup>. Therefore, T cells modified with CD79b-targeting TCRs can be a valuable addition to current immunotherapies.

T cells expressing high affinity TCRs recognizing self-antigens such as CD79b in the context of self-HLA molecules are deleted from the naïve T cell repertoire during thymic development by negative selection to prevent autoreactivity. In contrast, presentation of such self-antigens in the context of allogeneic HLA (alloHLA) can result in strong T cell responses as observed in the induction of PRAME-specific T cells after HLA-A\*0201-mismatched hematopoietic stem cell transplantation<sup>38</sup>. In an additional study we demonstrated that the high affinity TCRs of

these alloHLA-restricted T cells are single peptide-specific<sup>39</sup>. Using the concept of self-antigen presented in alloHLA, Wilms tumor antigen (WT1) reactive T cells have been generated by stimulating T cells from HLA-A2-negative donors with autologous B lymphocytes coated with HLA-A2 monomers loaded with WT1-derived peptide<sup>40</sup>. Additionally, antigen-specific T cells were obtained by stimulating T cells from HLA-A2-negative donors with autologous dendritic cells (DCs) pulsed with RNA to express HLA-A2 in parallel with a variety of targeted antigens<sup>41-43</sup>.

In this study, from HLA-A\*0201/B\*0701-negative healthy individuals we isolated 3 T cell clones targeting 3 distinct CD79b peptides presented in HLA-A\*0201 or HLA-B\*0702. CD79b-positive primary CLL, ALL and ALL cell lines were recognized by these T cell clones while no reactivity towards nonhematopoietic tissue was detected. However, low to intermediate recognition of CD14<sup>+</sup> monocytes, T cells and CD34<sup>+</sup> hematopoietic precursor cells (HPCs) could be observed. Although no cell surface CD79b was present on these cells, aberrant mRNA expression of CD79b could be detected in these cells.

## Results

### Identification of Naturally Processed and Presented CD79b Peptides

To identify MHC-presented CD79b peptides we adapted a previously described algorithm<sup>44</sup>. From a peptide elution library that was generated by eluting and sequencing MHC-bound peptides from human HLA-A2/B7-positive B-LCLs<sup>45</sup>, three peptides of CD79b were identified with mascot ion scores above 35 and netMHC<sup>46, 47</sup> binding scores lower than 250 nM (Table 1). Peptides CD79b<sub>9</sub> and CD79b<sub>173</sub> were newly identified. Peptide CD79b<sub>20</sub> had previously been eluted<sup>48, 49</sup>. All 3 peptides are encoded by each isoform of CD79b. Matching mass spectrometry (MS) fragmentation patterns of eluted and newly synthesized CD79b peptides verified correct identification (data not shown). Stable pMHC tetramers of CD79b<sub>20</sub> and CD79b<sub>173</sub> bound to HLA-A2 (CD79b<sub>20</sub>:A2 and CD79b<sub>173</sub>:A2, respectively) and CD79b<sub>9</sub> bound to HLA-B7 (CD79b<sub>9</sub>:B7) were generated by conjugating conventionally refolded pMHC monomers to streptavidine-labeled fluorophores.

### Generation of pMHC Tetramer<sup>+</sup> CD8<sup>+</sup> T Cell Lines by Tetramer-guided Enrichment

T cells binding to pMHC tetramers containing CD79b peptides presented in HLA-A2 or HLA-B7 were isolated from PBMCs of 13 HLA-A2/B7-negative healthy individuals using PE-labeled pMHC tetramers and MACS isolation. From  $6 \times 10^7$  to  $3 \times 10^8$  total PBMCs between 10,000-160,000 total cells were recovered in the pMHC tetramer-labeled fraction. Among the isolated cells, 7-21 % were CD8<sup>+</sup> T cells of which only a fraction stained positive for the pMHC tetramers (data not shown). Other cells included CD4<sup>+</sup> T cells, monocytes and B-cells. After two weeks of expansion, the generated T cell lines were subjected to a second enrichment using the same pMHC tetramers. Following two weeks of expansion, the presence of CD79b-

**Table 1.** Sequences and properties of CD79b-derived peptides.

	Peptide sequence	HLA-restriction	netMHC affinity (nM)*	Source
CD79b <sub>173</sub>	IIVPIFLLL	A*0201	177	Eluted <sup>45</sup>
CD79b <sub>9</sub>	VPSHWMVAL	B*0702	14	Eluted <sup>45</sup>
CD79b <sub>20</sub>	LLSAEPVPA	A*0201	112	Eluted <sup>45</sup> , previously described <sup>48,49</sup>

\*netMHC 3.4. server<sup>48,49</sup>

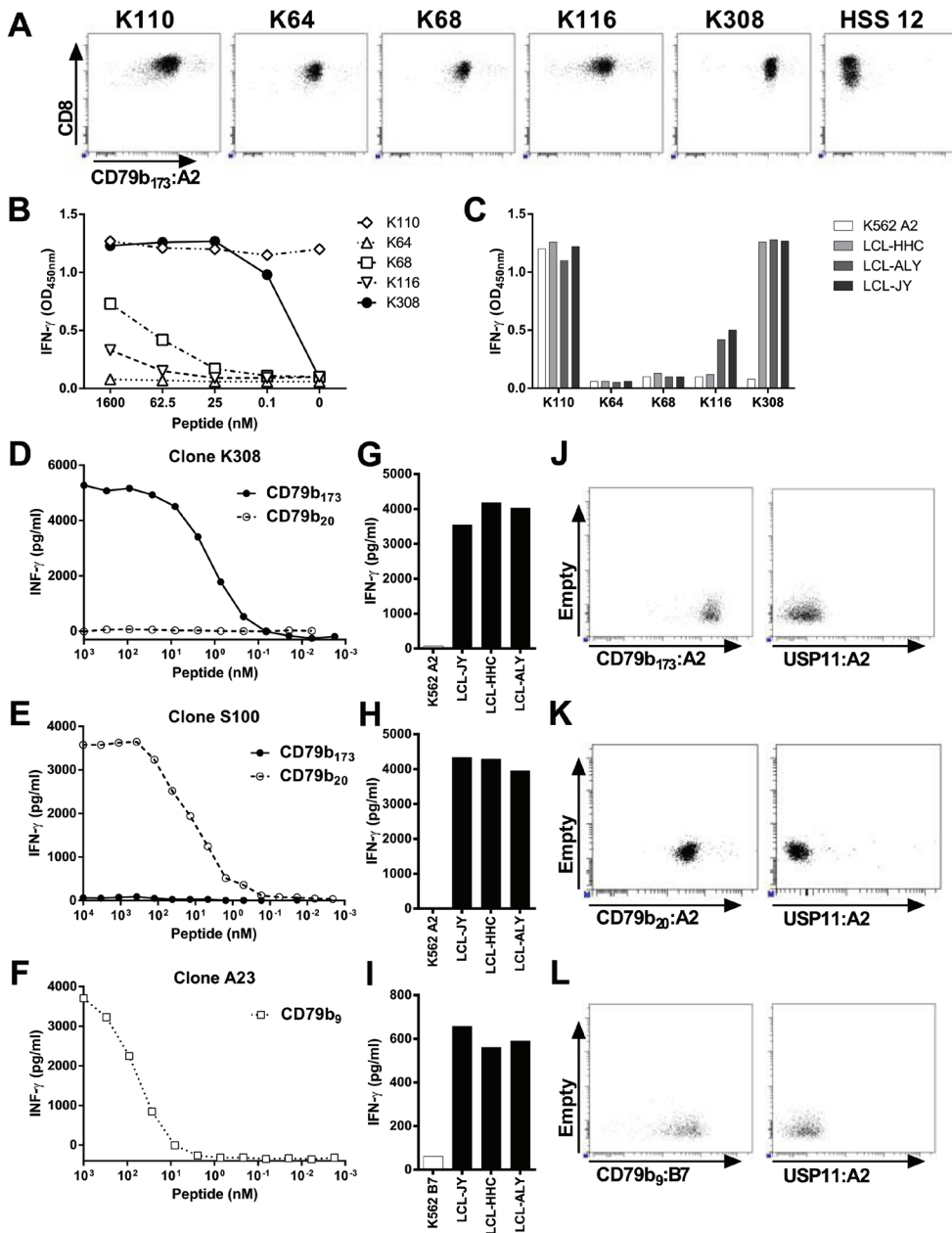
reactive T cells were determined by pMHC tetramer staining (Supplementary Table S1 and Supplementary Figure S1). In 11 of 13 individuals CD8<sup>+</sup> T cells positive for pMHC tetramer CD79b<sub>173</sub>:A2 were detected with frequencies of 0.02 to 23.8 % of CD8<sup>+</sup> T cells. CD8<sup>+</sup> T cells staining positive for CD79b<sub>20</sub>:A2 were present in three individuals with frequencies of 0.1 to 31.2 %. CD8<sup>+</sup> T cells staining positive for CD79b<sub>9</sub>:B7 were detected in three individuals with frequencies of 0.03 to 0.7 % of the CD8<sup>+</sup> T cell compartment.

In summary, CD8<sup>+</sup> T cells binding to pMHC tetramers presenting CD79b peptides in HLA-A2 or HLA-B7 could be isolated from the allogeneic repertoire of HLA-A2/B7-negative individuals.

### Identification of Three Distinct T Cell Clones Recognizing CD79b Peptides Presented in alloHLA

To analyze the functional activity of the CD8<sup>+</sup> T cell populations binding to CD79b pMHC tetramers, pMHC tetramer<sup>+</sup> CD8<sup>+</sup> T cells were clonally expanded using single cell-sorting. We sorted CD8<sup>+</sup> T cells binding to CD79b<sub>173</sub>:A2 from individuals GGT, REE, JTD, and PBI. CD8<sup>+</sup> T cell clones binding to CD79b<sub>20</sub>:A2 were obtained from individuals EBG and JTD. CD8<sup>+</sup> T cell clones binding to CD79b<sub>9</sub>:B7 were sorted from LJE. T cell clones were analyzed for CD79b peptide specificity and avidity by loading CD79b peptides on the CD79b-negative cell line K562 expressing HLA-A2 or HLA-B7 (K562-A2 or K562-B7, respectively). Recognition of endogenously processed and MHC-presented CD79b peptides was assessed on three HLA-A2/B7-positive B-LCLs which naturally express CD79b.

CD8<sup>+</sup> T cell clones binding to pMHC tetramer CD79b<sub>173</sub>:A2 demonstrated varying degrees of peptide specificity and levels of avidity. Figure 1A-C shows the tetramer staining and functional results of five representative clones. T cell clone K110 showed recognition of all three HLA-A2<sup>+</sup> B-LCLs, as well as unloaded K562-A2 cells illustrating non-CD79b specificity of the T cell clone. Clone K64 demonstrated no recognition of peptide-pulsed K562-A2 cells and no reactivity towards any CD79b-expressing B-LCL (Figure 1B and C, respectively). To exclude that absence of recognition could be attributed to failure to produce IFN- $\gamma$ , also GM-CSF secretion was assessed, but no GM-CSF production was observed (data not shown), suggesting an insufficient sensitivity of clone K64 to recognize MHC-bound CD79b<sub>173</sub> peptide. In contrast, T cell clones K68, K116 and K308 showed CD79b<sub>173</sub> peptide-dependent recognition of peptide-pulsed K562-A2 cells. Clone K308 demonstrated the highest peptide sensitivity (Figure 1B).



**Figure 1. pMHC tetramer<sup>+</sup> CD8<sup>+</sup> T cells exhibit different degree of CD79b peptide sensitivity and specificity.** T cells were clonally expanded by single cell-sorting of CD8<sup>+</sup> T cells binding to pMHC tetramers containing CD79b peptides. (A) Shown are pMHC tetramer staining of five representative clones (K110, K64, K68, K116 and K308) isolated using pMHC tetramer CD79b<sub>173</sub>:A2. Control include clone HSS12 specific for USP11-derived peptide FTWEGLYNV bound to HLA-A2. Shown are CD8<sup>+</sup> cells (B-C) The same T cell clones as in (A) were coincubated with K562-A2 cells loaded with decreasing concentrations of CD79b<sub>173</sub> peptide (B) or were cocultured with three HLA-A2/B7-positive B-LCLs endogenously expressing CD79b (C). Controls included CD79b-negative K562-A2 cells. (D-E) K562-A2 cells peptide-pulsed with decreasing concentration of peptide CD79b<sub>173</sub> or CD79b<sub>20</sub> as indicated were co-incubated with clone K308 isolated using pMHC tetramer CD79b<sub>173</sub>:A2 (D) or clone S100 isolated using pMHC tetramer CD79b<sub>20</sub>:A2 (E). (continuous on next page)



Corresponding with this high peptide sensitivity, clone K308 demonstrated reactivity towards all three B-LCLs whereas the recognition of three B-LCLs was low or absent for the low avidity clones K116 and K68 (Figure 1C) These results indicate that clone K308 exhibits sufficient avidity for CD79b<sub>173</sub> peptide to be able to efficiently recognize endogenously processed and presented CD79b in the context of HLA-A2 (Figure 1C, G).

Among the T cell clones isolated using pMHC tetramers CD79b<sub>20</sub>:A2 or CD79b<sub>9</sub>:B7 two T cell clones exhibited peptide-specific recognition of peptide-pulsed K562 cells. T cell clone S100 and A23 recognized peptide CD79b<sub>20</sub> and CD79b<sub>9</sub> when loaded on K562-A2 and K562-B7 cells, respectively (Figure 1E and F, respectively). Both T cell clones efficiently recognized all three B-LCLs (Figure 1H-I). Specificity for their cognate pMHC tetramers was confirmed for clones K308, S100 and A23 by FACS analysis (Figure 1J-L). None of the T cell clones stained with the control pMHC tetramer composed of HLA-A2 presenting peptide FTWEGLYNV derived from USP11. These data demonstrate that we have isolated high avidity peptide-specific T cell clones targeting CD79b peptides presented by alloHLA molecules. T cell clones K308, S100, and A23 were identified as the most avid clones targeting peptides CD79b<sub>173</sub>, CD79b<sub>20</sub>, and CD79b<sub>9</sub>, respectively.

### CD79b Transduction and CD79b Knockdown Verified CD79b Specificity of the T Cell Clones

To verify CD79b-dependent reactivity by T cell clones K308, S100, and A23, K562-A2 and K562-B7 cells were retrovirally transduced with CD79b. Clone K308 and S100 specific for HLA-A2-presented peptides CD79b<sub>173</sub> and CD79b<sub>20</sub>, respectively, demonstrated efficient recognition of K562-A2 engineered to express CD79b, whereas reactivity towards non-transduced K562-A2 or K562 carrying the wrong HLA restriction molecule was absent (Figure 2A). CD79b<sub>9</sub>-specific clone A23 efficiently recognized K562 simultaneously expressing HLA-B7 and CD79b, whereas no recognition was observed of non-transduced K562-B7. Recognition was also absent when HLA-A2 was expressed in CD79b-transduced K562 instead of HLA-B7. Furthermore, downregulation of CD79b with specific shRNA abrogated recognition of CD40L-stimulated CD19<sup>+</sup> B cells from HLA-A2<sup>+</sup> healthy individuals by clones K308 and S100 whereas the reactivity of control clone HSS12 specific for a peptide derived from the ubiquitously expressed gene USP11 presented in HLA-A2 was unaffected<sup>39</sup> (Figure 2B). Similar results were obtained for clone A23 were CD79b-specific knockdown abrogated recognition of LCL-JY (Supplementary Figure S2). In summary, CD79b-dependent and specific recognition was confirmed for T cell clones K308, S100, and A23.

(F) K562 cells retrovirally transduced with HLA-B7 (K562-B7) were peptide-pulsed with decreasing concentration of peptide CD79b<sub>9</sub> and coincubated with clone A23 isolated with pMHC tetramer CD79b<sub>9</sub>:B7. (G-I) Clone K308 (G), S100 (H) and A23 (I) were cocultured with HLA-A2 and HLA-B7-positive B-LCLs. CD79b-negative K562-A2 or K562-B7 cells were included as controls. IFN- $\gamma$  secretion was measured using standard ELISA. Axes were adjusted to maximum cytokine release measured. (J-L) K308 was stained with pMHC tetramer CD79b<sub>173</sub>:A2 (J), S100 with CD79b<sub>20</sub>:A2 (K) and A23 with CD79b<sub>9</sub>:B7 (L). Staining with an irrelevant pMHC tetramer USP11:A2 was used as control. Dot plots are shown with bi-exponential axis.

## CD79b-specific T Cell Clones Recognize B Cell Malignancies

Next, CD79b-specific T cell clones K308 and S100 were assessed for their potency to recognize a variety of primary B cell malignancies and malignant B cell lines. Clones K308 and S100 efficiently recognized HLA-A2-positive ALL cell lines (Figure 3A). Clone S100 also recognized 4 HLA-A2-positive primary CLL samples (Figure 3B). In addition, stimulation with primary HLA-A2-positive CLLs and ALLs samples resulted in strong IFN- $\gamma$  production of clone K308 (Figure 3C-D). No recognition of the HLA-A2-negative CLL sample ERV 8300 was observed (Figure 3D). Coincubation of the ALL cell lines or primary HLA-A2-positive ALL and CLL samples with clone K308 resulted in efficient lysis of the malignant cells (Figure 3E-F) indicating a cytotoxic effect of clone K308. No lysis of the HLA-A2-negative CLL sample ERV 8300 (Figure 3F) after coincubation with clone K308 was detected. Killing of the malignant cells was not observed for a CMV-specific control clone recognizing the irrelevant peptide NLVPMVATV bound to HLA-A2 (Figure 3E-F). These results indicate efficient recognition of CD79b-expressing HLA-A2-positive B cell malignancies by the CD79b-specific clones K308 and S100.

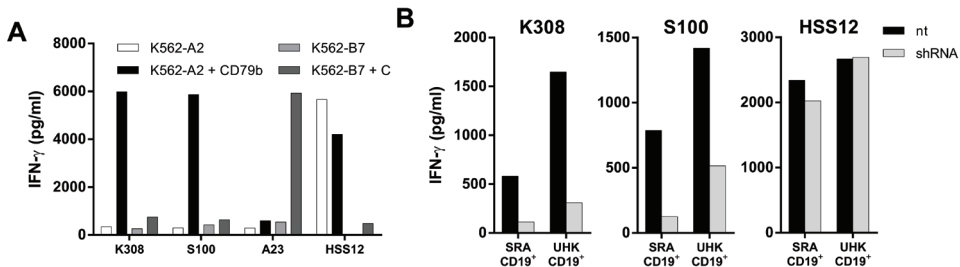
## No Reactivity Against Nonhematopoietic Tissue, But Unexpected Reactivity Against Different Hematopoietic Cell Subsets

To further validate that the recognition pattern of the CD79b-specific T cell clones is restricted to the B cell compartment, their reactivity towards healthy nonhematopoietic and hematopoietic cells was assessed. No IFN- $\gamma$  production was measured for K308 and S100 after overnight coculture with HLA-A2-positive fibroblasts or proximal tubular epithelial cells (PTECs) (Figure 4A). Recognition was also absent when fibroblast and PTECs were pretreated with IFN $\gamma$  for 4 days to simulate inflamed conditions. Control clone HSS12 recognized fibroblasts and PTECs under all conditions indicating the stimulatory capacity of the target cells. In addition, we assessed clone K308's reactivity towards hematopoietic cell populations by isolating different cell subsets from peripheral blood of HLA-A2-positive healthy individuals. As shown in Figure 4B, clone K308 did not recognize monocyte-derived immature and mature DCs. However, clone K308 exhibited intermediate reactivity against either activated or non-activated CD4<sup>+</sup> T cells, non-activated CD8<sup>+</sup> T cells and CD34<sup>+</sup> HPCs as well as low reactivity against CD14<sup>+</sup> monocytes (Figure 4B-D). Furthermore, coincubation of non-activated and activated B cells and T cells, and CD34<sup>+</sup> HPCs with clone K308 also led to their lysis which was not observed when target cells were co incubated with a CMV-specific T cell clone (Figure 4E-F). Similarly, clone S100 also recognized activated CD4<sup>+</sup> T cells (Figure 4G).

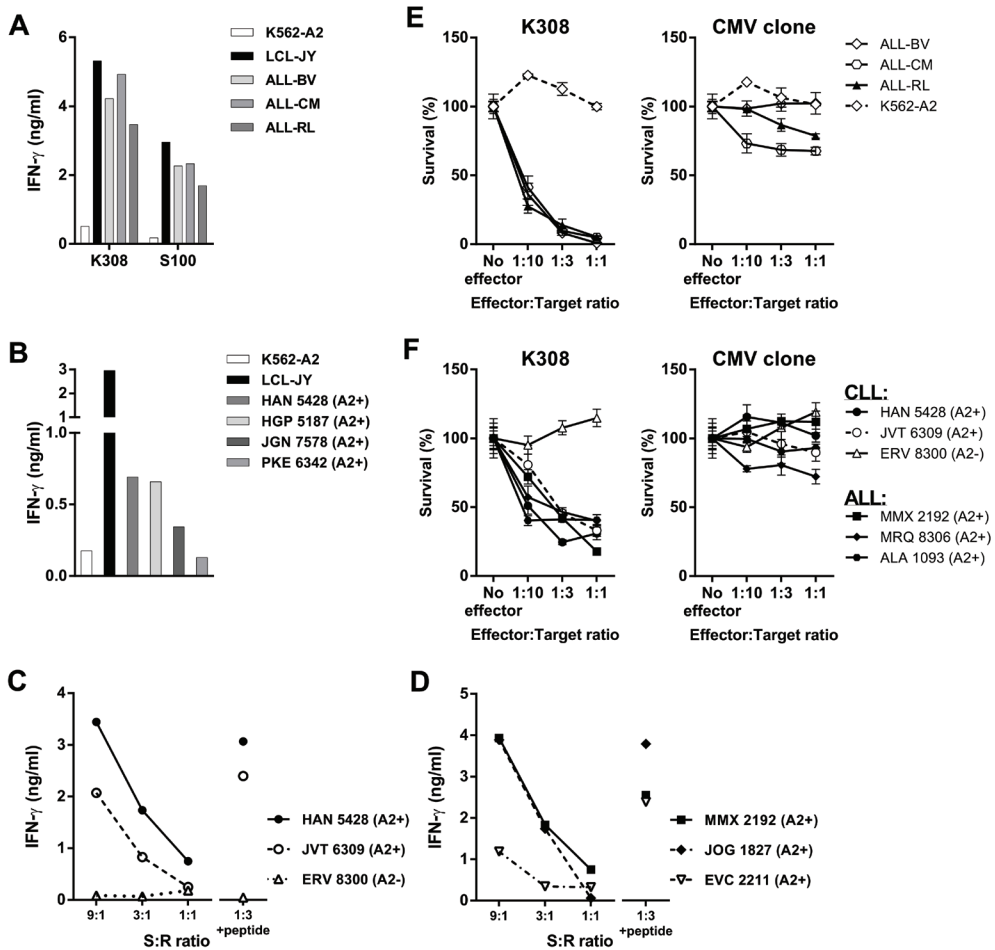
## Aberrant CD79b Expression in T Cells, HPCs and Monocytes Is Responsible for Reactivity of CD79b-specific T Cell Clones

To resolve the unexpected recognition of T cells, HPCs, and monocytes, we examined whether these cell populations demonstrated unreported CD79b cell surface expression. Staining of PBMCs with anti-CD79b antibody revealed no cell surface expression of CD79b on CD3<sup>+</sup> T

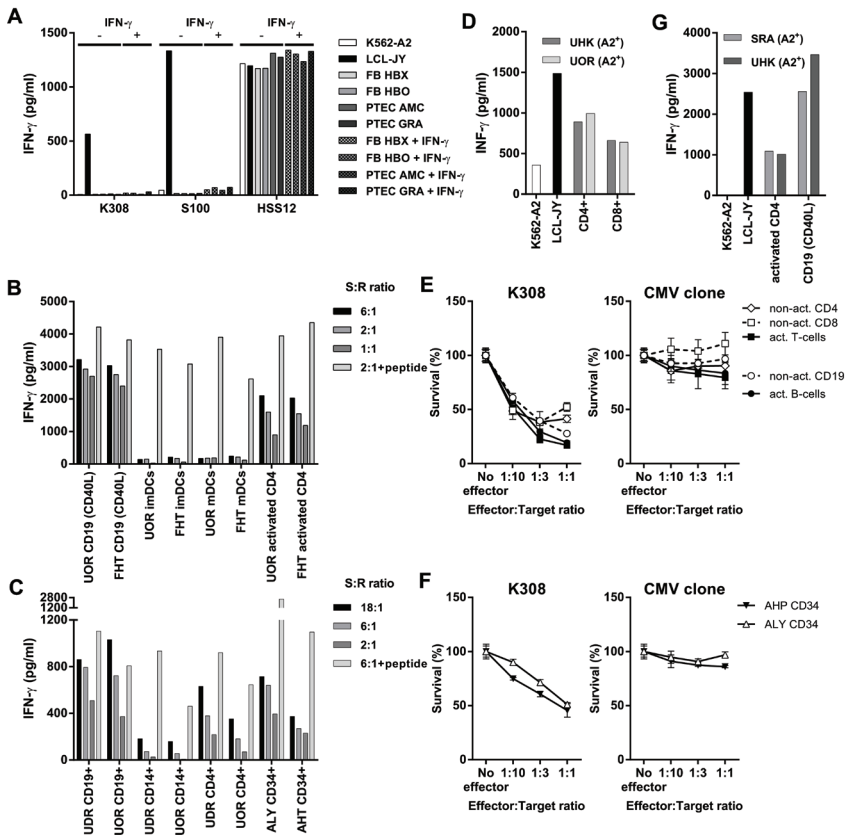
cells and CD14<sup>+</sup> monocytes (Figure 5A) Also, activated CD4<sup>+</sup> T cells and purified CD34<sup>+</sup> HPCs did not show detectable levels of cell surface CD79b, whereas CD79b was demonstrated on CD19<sup>+</sup> B cells (Figure 5A). To investigate whether gene expression of CD79b in cells outside of the B cell compartment occurred, we performed RT-qPCR. As expected, high expression of CD79b in CLL, ALL, B-LCL and healthy CD19<sup>+</sup> B cell samples was demonstrated by RT-qPCR (Figure 5B), whereas no expression could be detected for nonhematopoietic cells such as fibroblasts, keratinocytes, and PTECs under normal and IFN- $\gamma$  treated conditions. In addition, mature and immature DCs showed no or very low gene expression of CD79b (100-1,000fold lower expression compared to CD19<sup>+</sup> B cells). In activated CD4<sup>+</sup> T cells and CD34<sup>+</sup> HPCs the expression of CD79b was approximately only 10fold lower than in CD19<sup>+</sup> B cells correlating with intermediate recognition of these cell subsets. In weakly recognized CD14<sup>+</sup> monocytes and non-activated CD4<sup>+</sup> T cells the expression of CD79b was approximately 10-100fold lower compared to CD19<sup>+</sup> B cells. These results demonstrate a correlation between CD79b expression and the reactivity of the CD79b-specific clones, exhibiting strong reactivity against CD19<sup>+</sup> B cells, no recognition of fibroblasts and PTECs as well as immature and mature DCs but intermediate to low reactivity against T cells, HPCs and monocytes. In summary, aberrant gene expression of CD79b in CD4<sup>+</sup> T cells, HPCs and monocytes although undetectable at the cell surface, caused unexpected reactivity of the CD79b-specific T cell clones against these cell subsets.



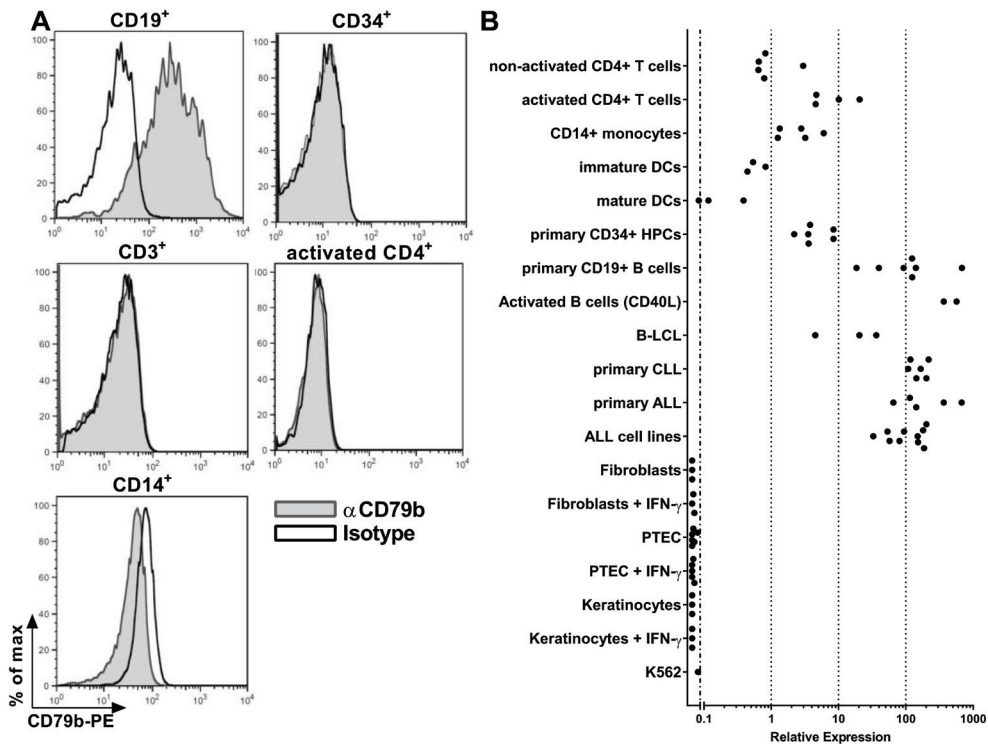
**Figure 2. CD79b expression and shRNA knockdown confirm CD79b specificity of isolated T cell clones.** (A) CD79b-negative K562 cells expressing either HLA-A2 (K562-A2) or HLA-B7 (K562-B7) were retrovirally transduced with codon-optimized CD79b (K562-A2 + CD79b or K562-B7 + CD79b) and sorted to high purity. CD79b-reactive T cell clones K308 (CD79b<sub>173</sub>:A2), S100 (CD79b<sub>20</sub>:A2) and A23 (CD79b<sub>9-17</sub>:B7) were coincubated with transductants. Control T cell clone HSS12 recognizes a peptide of the ubiquitously expressed gene USP11 in the context of HLA-A2. (B) CD40L activated CD19<sup>+</sup> B cells of two HLA-A2-positive healthy individuals (SRA CD19<sup>+</sup> and UHK CD19<sup>+</sup>) were transduced with CD79b-specific shRNA (shRNA) and selected by puromycin treatment or left untreated (nt) and coincubated with T cell clones K308, S100 or control clone HSS12. Clone K308 and S100 were cocultured at stimulator to responder (S:R) ratio of 2:1. Control clone HSS was cocultured at an S:R ratio of 10:1. IFN- $\gamma$  production was determined using standard ELISA. Antigen-presenting capacity of transduced and non-transduced target cells was controlled by clone HSS12.



**Figure 3. CD79b-specific T cell clones recognize malignant B cell lines and primary B cell malignancies.** (A) T cell clones K308 and S100 recognizing CD79b<sub>173</sub> and CD79b<sub>20</sub> peptide in the context of HLA-A2, respectively, were stimulated with three HLA-A2-positive ALL cell lines ALL-BV, ALL-CM and ALL-RL at an stimulator to responder (S:R) ratio of 10:1. Controls included CD79b-negative K562-A2 cells and CD79b-positive LCL-JY. (B) T cell clone S100 was stimulated with four primary HLA-A2-positive primary CLL samples at S:R ratio 10:1. (C and D) Clone K308 was tested against primary CLL (C) and primary ALL (D) samples at indicated S:R ratios. HLA-A2 genotype of primary samples is indicated in parenthesis. Controls included samples which were exogenously loaded with 100 nM CD79b<sub>173</sub> peptide (3:1+peptide). IFN- $\gamma$  production was assessed using standard ELISA. (E and F) Cytotoxicity of K308 was determined for ALL cell lines and primary CLL and ALL samples. PKH26GL-labelled ALL cell lines (E) or primary CLL and ALL samples (F) were co-incubated with K308 (K308) at different effector to target ratios for 17 hours. Living PKH26GL-labelled target cells were counted and analyzed by FACS, and the percent surviving cells was calculated (see Material and Methods). Controls include a CMV-specific T cell clone (CMV clone) recognizing irrelevant pp65-derived peptide NLVPMVATV presented in HLA-A2, and CD79b-negative K562-A2 cells. Experiment was carried out in triplicate showing mean $\pm$ SD.



**Figure 4. CD79b-specific T cell clones spare healthy nonhematopoietic tissue but demonstrate intermediate and low recognition of T cells, HPCs and monocytes.** CD79b-specific T cell clones were assessed for their reactivity towards healthy nonhematopoietic tissue and hematopoietic cell subsets. **(A)** Recognition of HLA-A2-positive fibroblasts (FB) and proximal tubular epithelial cells (PTECs) untreated (-) or pretreated with IFN- $\gamma$  (+) for 4 days was assessed after coculturing with CD79b-reactive T cell clones K308 and S100 for 18h at stimulator to responder (S:R) ratio of 5:1. Controls included CD79b-negative K562-A2 cells, CD79b-positive LCL-JY and T cell clone HSS12 recognizing a peptide of the ubiquitously expressed USP11 presented in HLA-A2. **(B and C)** Reactivity towards HLA-A2-positive healthy hematopoietic cells was assessed by coculturing clone K308 with cultured (B) and non-activated (C) cell subsets at indicated S:R ratios for 18 hours. Controls included peptide-pulsing of stimulators at a concentration of 100 nM peptide (2:1+peptide or 6:1+peptide). CD19<sup>+</sup> B cells were cocultured with irradiated CD40L-expressing mouse fibroblasts for six days prior to the experiment (CD19 (CD40L)). Immature dendritic cells (imDCs) and mature DCs (mDCs) were CD14<sup>+</sup> monocyte derived (see Supplementary Methods). Activated CD4<sup>+</sup> were generated from CD4<sup>+</sup> cells that were treated with 0.8 ng/ml PHA and cultured for 10 days. Non-activated CD19<sup>+</sup>, CD14<sup>+</sup>, CD4<sup>+</sup> and CD34<sup>+</sup> cells were isolated from peripheral blood using MACS isolation. **(D)** Clone K308 was stimulated with primary CD4<sup>+</sup> or CD8<sup>+</sup> cells in a S:R ratio of 6:1. **(E and F)** Cytotoxicity of clone K308 was determined for non-activated CD4 and CD8 T cells and activated T cells (containing CD4 and CD8), and activated and non-activated CD19 B-cells (E), and CD34<sup>+</sup> HPCs (F). PKH26GL-labelled target cells were coincubated with K308 (K308) at different effector to target ratios for 17 hours. Living PKH26GL-labelled target cells were counted and analyzed by FACS and the percent surviving cells was calculated (see Material and Methods). Control included a CMV-specific T cell clone (CMV clone) recognizing irrelevant pp65-derived peptide NLVPMVATV presented in HLA-A2. Experiment was carried out in triplicate showing mean $\pm$ SD. **(G)** T cell clone S100 recognizing peptide CD79b<sub>20</sub> in HLA-A2 was stimulated with activated CD4<sup>+</sup> T cells or CD40L-activated CD19<sup>+</sup> B cells from two HLA-A2-positive individuals (SRA and UHK) in an S:R ratio of 10:1. IFN- $\gamma$  secretion was assessed by standard ELISA.



**Figure 5. Aberrant CD79b gene expression in T cells, HPCs and monocytes in the absence of cell surface expression.** (A) PBMCs were stained with CD79b-specific antibody ( $\alpha$ CD79b) or isotype control (Isotype) in combination with lineage specific markers as indicated. Histograms show CD79b expression after gating on B cells (CD19<sup>+</sup>), T cells (CD3<sup>+</sup>) and monocytes (CD14<sup>+</sup>). CD34<sup>+</sup> HPCs were isolated from G-CSF mobilized blood. Activated CD4<sup>+</sup> T cells were stimulated with PHA and irradiated autologous feeders and cultured for 10 days. (B) CD79b mRNA expression was determined for indicated cell subsets by qRT-PCR. Expression was normalized to average CD79b expression in primary CD19<sup>+</sup> B cells which was set to 100. Dots indicate individual samples.

## Discussion

In this study, we aimed to isolate T cells expressing TCRs specific for BCR associated protein CD79b that is expressed on normal and malignant B cells<sup>14-16</sup>. MHC-presented peptides of CD79b were identified from peptide elution studies<sup>45, 48, 49</sup>. By exploiting the immunogenicity of alloHLA molecules, three distinct T cell clones derived from different HLA-A\*0201/B\*0702 healthy individuals were isolated targeting distinct peptides of CD79b presented in HLA-A\*0201 or HLA-B\*0702. The CD79b-specific clones efficiently recognized primary CLL, ALL and ALL cell lines whereas no reactivity towards nonhematopoietic cells and professional antigen-presenting cells (APCs) was detected. However, unexpected reactivity towards monocytes, HPCs and T cells was observed. Aberrant intracellular CD79b expression in those cell subsets was detected in the absence of cell surface CD79b.

Although all T cell clones were isolated by binding to pMHC tetramers containing CD79b

peptides, CD79b specificity was not observed in all cases. Various T cell clones lacked specificity for CD79b peptides, indicated by recognition of CD79b-negative K562-A2 or K562-B7 cells. This reactivity is probably the result of crossreactivity with other alloHLA-bound peptides and resembles the reactivity previously observed for WT1-specific T cells derived from the allogeneic repertoire<sup>50</sup>. Other isolated T cell clones showed no reactivity towards peptide-loaded stimulator cells and CD79b-expressing B-LCLs suggesting insufficient T cell avidity. In contrast, we also isolated pMHC tetramer<sup>+</sup> T cell clones with variable sensitivity for the CD79b peptide-loaded stimulators. Among these T cell clones, only the most avid clones were capable to recognize endogenously processed peptide. We have previously illustrated<sup>51</sup> that not pMHC tetramer binding but TCR-ligand  $k_{off}$ -rate as measured by decay of pMHC:TCR interaction correlates with functional characteristics. Assessment of sensitivity and specificity for MHC-bound CD79b peptides on a clonal level was therefore imperative to select for the most avid and CD79b-specific clones.

For three high avidity T cell clones, we confirmed CD79b specificity by CD79b transduction into K562 cells and abrogation of recognition of B-LCLs and CD19<sup>+</sup> B-cells after shRNA-mediated CD79b-specific knockdown. Furthermore, absence of reactivity towards CD79b-negative cells such as nonhematopoietic cell lines pretreated with IFN- $\gamma$  and professional antigen-presenting cells indicated that CD79b-independent activation of our CD79b-specific T cell clones does not easily occur. However, recognition of T cells, HPCs and monocytes was observed which correlated with low aberrant intracellular CD79b expression in these cells. Although we cannot formally exclude the recognition of peptide look-alikes or mimitopes in these cells, the strong CD79b-dependency of our CD79b-specific T cell clones argues otherwise.

Recognition of primary B cell malignancies by our T cell clones suggested that the expressed CD79b-specific HLA-A\*0201 or B\*0702-restricted TCRs could be used for TCR gene transfer approaches to treat HLA-A\*0201 or B\*0702-positive patients suffering from B cell malignancies. However, we also observed recognition of monocytes, T cells and HPCs. In contrast to B cell depletion, long-term monocytopenia, T cell and HPC ablation is not tolerable from a clinical perspective. The high avidity CD79b-specific T cells recognizing a self-antigen such as CD79b when presented in HLA-A2 or HLA-B7 were isolated from HLA-A2/B7-negative individuals. Negative selection during thymic development does not deplete these high avidity T cells in these individuals since the HLA-A2 and HLA-B7 restriction molecules are not present. In HLA-A2 and HLA-B7-positive individuals, however, the elimination of such high avidity CD79b-specific T cells is crucial to prevent loss of B cells, monocytes, and HPCs due to autoreactivity. Furthermore, the CD79b expression in HLA-A2/B7-positive T cells would also cause fratricide<sup>41</sup> among T cells; T cells would simultaneously express the correct HLA restriction molecule as well as the CD79b antigen recognized by their TCR. In concordance, we have previously shown that self-HLA-restricted PRAME-specific T cells possess insufficient avidity to recognize endogenously expressed PRAME<sup>38</sup>. Therefore, it is very unlikely that such high avidity CD79b-specific T cells as demonstrated in this study could be isolated from the autologous repertoire of HLA-A2 and HLA-B7 individuals.

In conclusion, exploiting the immunogenicity of alloHLA molecules is an effective and attractive strategy to overcome self-tolerance towards self-antigens. TCRs raised from alloreactive T cells can meet the requirements for high affinity interaction with self-antigens while preserving peptide specificity and HLA-restriction. Their identification can be readily achieved by pMHC tetramer based isolation but requires functional assessment on a clonal level. Aberrant expression of CD79b in monocytes, T cells and HPCs eliminated CD79b as a suitable target for TCR-based immunotherapy to treat B cell malignancies. However, CD79b may still be an attractive target for CAR or antibody-based immunotherapies since no cell surface expression on monocytes, T cells and HPCs was observed.

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## Materials and Methods

### Culture Conditions and Cells

Peripheral blood mononuclear cells (PBMCs) were obtained from different individuals, after their informed consent, by isolation using Ficoll-gradient centrifugation and were cryopreserved. T cells were cultured in T cell medium consisting of Iscove's Modified Dulbecco's Medium (IMDM, Lonza, Basel, Switzerland) supplemented with 100 IU/ml IL-2 (Proleukine, Novartis Pharma, Arnhem, The Netherlands), 5 % fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA, USA) and 5 % human serum. Primary hematopoietic cell subsets were purified from cryopreserved PBMCs of HLA-A\*02:01 and B\*07:02-positive healthy donors using anti-CD4, anti-CD8, anti-CD14, anti-CD19 or anti-CD34 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of isolated cells was assessed using FACS analysis and cells were only used in experiments if the purity exceeded 95 %. Immature and mature dendritic cells (DCs) were differentiated *in vitro* from isolated CD14+ cell populations as previously described<sup>38</sup>. Activated CD4+ T-cells were generated by stimulating purified CD4+ T-cells with irradiated (35 Gy) PBMCs in a 1:5 ratio in T-cell medium supplemented with 0.8 µg/ml phytohemagglutinin (PHA, Remel, Lenexa, KS, USA) for 10 days. Activated CD19+ B-cells were generated by co-culturing CD19+ cells on CD40L-transduced irradiated (70 Gy) mouse-fibroblasts for 7 days in IMDM supplemented with 2 ng/ml IL-4 (Schering-Plough, Kenilworth, NJ, USA) and 10 % human serum. K562 cells expressing HLA-A2 (K562-A2) were previously described<sup>52</sup>. K562



cells expressing HLA-B7 (K562-B7) were generated by retroviral transduction with HLA-B7. Acute lymphoblastic leukemia (ALL) cell lines were previously described<sup>53</sup>. Fibroblasts and keratinocytes were isolated and cultured as previously described<sup>38</sup>. Proximal tubular epithelial cells (PTECs) were a kind gift of Prof. Cees van Kooten (Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands) and were cultured as previously described<sup>54</sup>. Fibroblasts, keratinocytes and PTECs were cultured in the presence or absence of 200 IU/ml IFN- $\gamma$  for four days. The Leiden University Medical Center review board approved the use of human material, and research was conducted in accordance with the Declaration of Helsinki.

### Generation of Peptide-MHC Complexes

All peptides were synthesized in-house using standard Fmoc chemistry. Recombinant HLA-A2 and HLA-B7 heavy chain and human  $\beta_2m$  light chain were in-house produced in *Escherichia coli*. Major histocompatibility complex (MHC) class I refolding was performed as previously described with minor modifications<sup>55</sup>. MHC class I complexes were purified by gel-filtration using HPLC. Peptide-MHC (pMHC) tetramers were generated by labeling biotinylated pMHC monomers with streptavidine-coupled phycoerythrin (PE, Invitrogen, Carlsbad, CA, USA) or Quantum dots (Qdots, Invitrogen) Q585, Q605, Q655 and Q705. Complexes were stored at 4 °C and spun at 17,000 g for 2 min prior to use.

### Isolation of CD79b-specific T Cell Clones Using pMHC Tetramers

T cells binding to pMHC tetramer containing CD79b peptides were isolated from cryopreserved PBMCs from healthy HLA-A\*02:01 and HLA-B\*07:02-negative individuals following a previously described protocol<sup>44</sup>. PBMCs were incubated with PE-labeled pMHC tetramers for 1 hour at 4 °C. Cells were washed twice and incubated with anti-PE magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). PE-labeled cells were isolated by MACS on a LS column (Miltenyi Biotec) according to the manufacturer's instruction. Positive fractions were cultured at 5,000 cells/well in 96-well round-bottom culture plates containing  $5 \times 10^4$  irradiated (35 Gy) autologous PBMCs and 11,000 anti-CD3/CD28-beads (Invitrogen) in 100  $\mu$ l T cell medium. After 2 weeks of expansion, CD79b-reactive T cell clones were obtained from pMHC tetramer enriched T cell populations by sorting CD8<sup>+</sup> T cells stained with PE-labeled pMHC tetramers containing CD79b peptides and an antibody against CD8 (Invitrogen/Caltag, Buckingham, UK) combined with antibodies against CD4, CD14, and CD19 (BD Pharmingen, San Jose, CA, USA). Cells were first stained with PE-labeled pMHC tetramers for 1 h at 4 °C before antibodies were added for an additional 15 min at 4 °C. Single pMHC tetramer<sup>+</sup> CD8<sup>+</sup> T cells were sorted into 96-well round-bottom culture plates containing  $5 \times 10^4$  irradiated allogeneic PBMCs in 100  $\mu$ l T cell medium supplemented with 0.8  $\mu$ g/ml PHA. Cell-sorting was performed on a FACSAria III (BD Biosciences, San Jose, CA, USA).

## FACS Analysis

FACS was performed on a LSRII (BD Biosciences) and analyzed using Diva Software (BD Biosciences) or on a FACS Calibur (BD Biosciences) and analyzed using FlowJo Software (TreeStar, Ashland, OR, USA). T cell lines generated by MACS enrichment using pMHC tetramers, and T cell clones were analyzed for pMHC tetramer<sup>+</sup> CD8<sup>+</sup> T cells by staining with pMHC tetramers CD79b<sub>173</sub>:A2 labeled with PE or Q705, CD79b<sub>20</sub>:A2 labeled with Q705 or Q655, CD79b<sub>9</sub>:B7 labeled with Q605 or Q585, and an Alexa700-conjugated antibody against CD8 (Invitrogen/Caltag) combined with FITC-labelled antibodies against CD4, CD14, and CD19 (BD Pharmingen). Cells were first incubated with a mixture of pMHC tetramers (0.2 µg/ml per pMHC tetramer) for 15 min at 37 °C before antibodies were added and incubated for an additional 15 min at 4 °C. PBMCs, purified hematopoietic cell subsets or activated cells were stained with antibodies against CD79b (BD Pharmingen, clone 3A2-2E7) or isotype control in combination with CD3, CD4, CD14, CD19, CD34 (BD Pharmingen) or CD271 (NGF-R, Sanquin, Uden, The Netherlands) at 4 °C for 15 min.

## Functional Analysis

Stimulator cells were peptide-pulsed at various peptide concentrations for 30 min at 37 °C. Responder T cells and peptide-pulsed or unloaded stimulator cells were co-incubated at various responder to stimulator ratios. After 18 h coincubation, supernatants were harvested and IFN-γ production was measured by enzyme-linked immunosorbent assay (ELISA, Sanquin Reagents, Amsterdam, The Netherlands).

## Cytotoxicity Assay

Adapted from Jedema *et al.*<sup>56</sup>, 20,000 PKH26GL-labelled (Sigma-Aldrich, St. Louis, MO, USA) target cells were coincubated with T cells at various effector:target ratios in 100 µl T cell medium for 17 hours. After coincubation, cells were stained with Sytox Blue dead cell stain (Invitrogen/Caltag) in a final concentration of 1 µM for 5 min. 10 µl Flow-count fluorospheres (Beckman Coulter, Indianapolis, IN, USA) were added and samples were analyzed using FACS. For each sample, 2,500 Flow-count fluorospheres were acquired and the percent surviving cells was calculated as follows: (PKH26GL-labelled targets in the presence of effector cells)/(PKH26GL-labelled targets in absence of effector cells)×100.

## Transduction with CD79b Retroviral Vector and CD79b-specific shRNA

CD79b (Accession Number NM\_000626.2\_variant1) was codon-optimized (GeneArt, Life Technologies) and expressed on a MP71 retroviral backbone in combination with NGF-R. CD79b-targeting short-hairpin RNAs (shRNA) TRC 057 648 and TRC 057 651 (Sigma Aldrich, St. Louis, MO, USA) were a kind gift of Prof. Rob Hoeben (Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands). shRNAs were expressed

on retroviral backbone pLKO.1 or pGreenPuro (Systembiosciences, Mountain View, CA, USA), both containing a puromycin resistance gene. For retrovirus production, Phoenix-A cells were plated at  $4 \times 10^4$  cells/cm<sup>2</sup> in T25 flasks. After 24 h, cells were transfected with 4 µg retroviral vector DNA and 2 µg M57 vector DNA using Fugene HD transfection reagent (Roche, Basel, Switzerland). Supernatant containing retroviral particles was harvested at 48 h and 72 h post transfection and stored at -80 °C. For transduction, retroviral supernatant was loaded on 24-well non-tissue-culture treated plates which had been coated with 30 µg/ml retronectin (Takara, Shiga, Japan) and blocked with 2 % human serum albumin (Sanquin Reagents). Viral supernatant was spun down at 2,000 g for 20 min at 4 °C.  $1 \times 10^5$  K562-A2 or K562-B7 cells were added to CD79b retroviral supernatant and incubated for 18 h. High purity bulk CD79b-transduced K562-A2 and K562-B7 cell populations were obtained by sorting cells stained with an antibody against NGF-R.  $1 \times 10^5$  cells of Epstein-Barr virus (EBV) transformed lymphoblastic cell lines (LCLs) or  $3 \times 10^5$  CD40L activated B cells were added to retroviral supernatant encoding for CD79b-targeting shRNA and incubated for 18 h. Transduced cells were selected by supplementing medium with 2-10 µg/ml puromycin (Calbiochem, San Diego, CA, USA) for two days. Optimal puromycin concentration for each cell type was determined by escalating dose experiments.

### **Real-time Quantitative Polymerase Chain Reaction (RTqPCR)**

Total RNA was isolated using the RNAqueous Micro-Kit and Small Scale Kit (Ambion, Life Technologies) for a maximum of  $0.5 \times 10^6$  and  $10 \times 10^6$  cells, respectively, following the manufacturer's instructions. Total RNA was converted to cDNA using M-MLV reverse transcriptase (Invitrogen). CD79b expression was measured on the Roche Lightcycler 480 (Roche) using Fast Start TaqDNA Polymerase (Roche) and EvaGreen (Biotium, Hayward, CA, USA) with forward primer 5'-CCAGGCTGGCGTTGTCTCCTG-3' and reverse primer 5'-GGTACCGTCCTCCGATCTGGC-3'.

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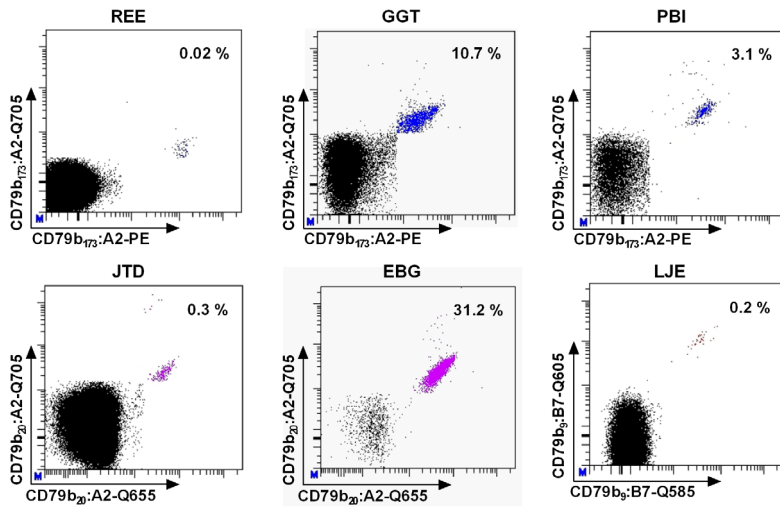
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## Supplementary Information

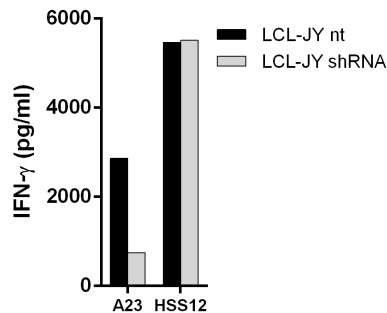
**Supplementary Table S1.** Presence (+) of pMHC tetramer<sup>+</sup> CD8<sup>+</sup> T cells after pMHC tetramer enrichment among total CD8<sup>+</sup> T cells. T cells were isolated using CD79b-specific pMHC tetramers and expanded before analysis.

Donor	CD79b <sub>173</sub>	CD79b <sub>20</sub>	CD79b <sub>9</sub>
GGT	+		+
BBD	+		
GSQ			+
AVZ			
GCF	+		
GMZ	+		
EWV	+		
REE	+		
EBG	+	+	
CVI	+	+	
JTD	+	+	
PBI	+		
LJE	+		+





**Supplementary Figure S1. Detection of potential CD79b-reactive CD8<sup>+</sup> T cell populations by pMHC tetramer staining.** CD8<sup>+</sup> T cell populations binding to pMHC tetramers presenting CD79b peptides in HLA-A2 or HLA-B7 were isolated from PBMCs of HLA-A2/B7-negative healthy individuals and expanded for two weeks. T cells were incubated with pMHC-tetramers dually encoded with different fluorophores as indicated on axes and gated on CD8<sup>+</sup> T cells. pMHC tetramer<sup>-</sup> CD8<sup>+</sup> T cells are shown in black. CD8<sup>+</sup> T cells binding to only one or more than two fluorophore-labeled pMHC tetramers were excluded from analysis. Numbers in upper right-hand corner of dot plots indicate percentages of pMHC tetramer<sup>+</sup> T cells among total CD8<sup>+</sup> T cells. Dot plots are shown with bi-exponential axis.



**Supplementary Figure S2. CD79b-specific shRNA knockdown abrogates recognition of B-LCL by CD79b-specific clone A23.** LCL-JY was transduced and selected by puromycin treatment to express a shRNA specifically targeting CD79b mRNA for degradation. shRNA-transduced B-LCL (LCL-JY shRNA) and untransduced B-LCL (LCL-JY nt) were co-incubated with T cell clone A23 (left) or control clone HSS12 (right) at stimulator to responder (S:R) ratio 9:1.

