Identification of a 40S Ribosomal Protein S4–Derived H-Y Epitope Able to Elicit a Lymphoblast-Specific Cytotoxic T Lymphocyte Response

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ABSTRACT

Purpose: **The superior graft-versus-leukemia (GVL) effect of female-to-male stem cell transplantation is partially independent from the concomitant graft-versus-host reactivity. However, the antigenic basis of this selective GVL response remains enigmatic, because no H-Y antigens with hematopoietic-restricted expression were identified. In this study, we report a novel H-Y epitope that is preferentially recognized on activated proliferating lymphocytes.**

Experimental Design: **We generated a CTL clone YKIII.8 that showed reactivity toward male B*5201$^+$ CD40-activated B cells, EBV-lymphoblastoid cell lines, and phytohemagglutinin-activated T-cell blasts but little or no reactivity toward fibroblasts, CD14$^+$ cells, or unstimulated B and T cells. The antigen recognized by YKIII.8 was identified by screening of a cDNA expression library, and its pattern of expression was investigated.**

Results: **cDNA of the male isoform of 40S ribosomal protein S4 was found to encode the antigenic peptide TIRYPDPVI, which was recognized by YKIII.8. Western blot analysis showed that rapidly proliferating cells overexpress the RPS4 protein in comparison with nonrecognized cell subsets. Retroviral transfer of YKIII.8 T-cell receptor resulted in preservation of the lymphoblast-specific reactivity pattern.**

Conclusion: **Our findings suggest that CTL specific to certain epitopes of ubiquitously expressed H-Y antigens may specifically target lymphoblasts, contributing to the selective GVL effect of female-to-male stem cell transplantation.**

INTRODUCTION

Allogeneic stem cell transplantation, donor lymphocyte infusion, and nonmyeloablative stem cell transplantation have been proven to be successful treatment strategies for patients with a variety of hematologic malignancies (1–4). The effectiveness of these therapeutic approaches is based on the graft-versus-leukemia (GVL) effect, an immune response of donor T lymphocytes toward malignant cells of the patient. However, the GVL effect is closely associated with graft-versus-host disease (GVHD), a frequent complication occurring as a result of donor T-cell reactivity to healthy tissues of the patient (5).

In a HLA-matched setting, both GVL and GVHD are caused mostly by T-cell responses to minor histocompatibility antigens (mHag), which are peptides derived from polymorphic intracellular proteins that are different in donor and patient. Disparity in mHags between patient and donor was shown to have a significant impact on the severity of GVHD and the clinical outcome of leukemia (6, 7). H-Y antigens represent a separate class of mHags. These antigens are encoded by genes located on the Y chromosome and are absent in females. H-Y antigens can induce both T-cell and antibody immune response after sex-mismatched allogeneic stem cell transplantation and during pregnancy (8, 9). Interestingly, male recipients of transplants from female stem cell transplantation donors are at the lowest risk of relapse in comparison with other patients even after controlling for GVHD as a time-dependent covariate (10). This suggests that anti-H-Y responses produce a selective GVL effect distinct from the one contributed by GVHD. Therefore, H-Y antigens should exist, which elicit immune responses selectively targeting leukemic cells. Four H-Y genes (*SMCY*, *DBY*, *UTY*, and *DFFRY*) were identified thus far as targets of CTL responses (11–14). Although UTY-specific CTL showed selective lytic activity toward hematopoietic cells and not fibroblasts or bone marrow stromal cells (15), none of the H-Y antigens identified thus far has a hematopoietic lineage-restricted pattern of expression or is overexpressed in leukemic cells. Therefore, identification of H-Y antigens capable of inducing immune responses specifically targeting cells of hematopoietic origin is important.

In this study, we report identification of a novel HLA-B*5201-restricted epitope of the male isoform of 40S ribosomal protein S4 (RPS4Y), which is able to induce a CTL response targeting proliferating lymphocytes.

MATERIALS AND METHODS

Cell Culture. Activation and expansion of B cells were done with the use of the 293-CD40-sCD40L system.[1] Briefly, B cells were expanded from peripheral blood mononuclear cells (PBMC) by incubation with 1.2×10^4 irradiated (75 Gy) 293 cells expressing both membrane-bound and soluble CD40L in presence of 10 ng/mL interleukin (IL)-10 (Peprotech, London, United Kingdom) and 200 units/mL IL-4 (Peprotech). B cells were harvested every 3 to 4 days and plated again at 6×10^4

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per well of a 24-well plate (Nunc, Rochester, NY). B and T cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated pooled human AB serum, 100 units/mL penicillin (Life Technologies, Gaithersburg, MD), 100 μg/mL streptomycin (Life Technologies), and 5×10^{-5} mol/L β-mercaptoethanol (Merck, Haarlem, the Netherlands). The amphotropic Phoenix packaging cell line, fibroblasts, and 293-EBNA-B7 cell lines were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated FCS (Integro BV, Leuvenheim, the Netherlands) and β-mercaptoethanol. In some experiments, fibroblasts were cultured in presence of 5 ng/mL IFN-γ (Peprotech) and 200 units/mL tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN) for 2 days before the test. All other cell lines were cultured in RPMI 1640 with 10% FCS and β-mercaptoethanol.

YKIII T-Cell Line and YKIII.8 T-Cell Clone. The YKIII T-cell line was generated through repetitive stimulation of T cells of a female donor with chronic myelogenous leukemia (CML) cells and then later with EBV-lymphoblastoid cell lines (EBV-LCL) of her HLA-identical (HLA-A1, A32, B52, B35, Cw4, DR2, DR7, and DR53) brother. The male CML patient achieved a complete remission after the bone marrow transplantation while suffering from the low-grade graft-versus-host disease. The female donor had given birth to three sons and had a history of blood transfusions.

The T-cell clone YKIII.8 was generated by plating the YKIII T-cell line under limiting dilution conditions. YKIII.8 clone was restimulated once every 2 weeks in 96-well round-bottomed plates (Nunc) with 0.5×10^6 T cells per plate, 1×10^6 irradiated (50 Gy) patient EBV-LCL, and 2×10^6 irradiated (25 Gy) PBMC pooled from three donors in the presence of 1 μg/mL phytohemagglutinin (PHA; Murex Diagnostics, Dartford, United Kingdom) and 300 IU/mL IL-2 (Proleukin, Chiron, Amsterdam, the Netherlands). One week after stimulation, T cells were harvested and cultured for 1 week at a cell concentration of 0.5×10^6 to 1×10^6 per milliliter in the presence of 300 IU/mL IL-2. Five to 7 days after harvesting, T cells were used for functional assays.

Cloning of HLA-B Molecules. RNA was isolated from the EBV-LCL of patient origin (EBV_p) with Trizol reagent (Invitrogen). Reverse transcription-PCR was done with HLA-B-specific primers 5′-GGGGTCGACATGCGGGTCAC-3′ and 5′-GGGGCGGCCGCTCAAGCTGTGAGAGA-3′. The PCR product was purified with the Qiagen Gel Extraction kit (Qiagen, Hilden, Germany) and digested with *Sal*I and *Not*I restriction endonucleases (New England Biolabs, Beverly, MA). The resulting fragment was subcloned in the pEGFP-N1 vector (Clontech, Palo Alto, CA) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Subsequently, HLA-B*3508 and HLA-B*5201 were cloned separately into the retroviral pMX vector (16).

Construction and Screening of the cDNA Library. Total RNA was isolated from the EBV_p with Trizol reagent. Enrichment for polyadenylated RNA was done with an oligo(dT)-cellulose column (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized with the SuperScript Choice System (Invitrogen) using a mixture of oligo(dT) (17) and a random primer 5′-CCCGCGGCCGC*NNNNNN*-3′ (where *N* = G, A, T, or C) containing *Not*I restriction site at 5′ end. The cDNA was ligated to phosphorylated *Hin*dIII adaptors, digested with *Not*I enzyme, and fractionated on the cDNA size fractionation column (Invitrogen). cDNA fraction of the largest size was cloned in *Hin*dIII and *Not*I sites of the expression vector pCEP4 (Invitrogen). *Escherichia coli* ElectroMAX DH5α-E competent cells were transformed by electroporation with ligation products. The library was divided in 720 pools of ~50 cDNA clones each. After 4 hours of amplification, plasmid DNA was extracted from individual pools with the QIAprep Spin Miniprep kit (Qiagen). 293-EBNA-B7-B*5201 cells plated in flat-bottomed 96-well plates (4×10^4 cells per well) 24 hours in advance were transfected in duplicates with 1.2 μL Lipofectamine (Invitrogen) and 100 ng DNA from the corresponding pool of the cDNA library. After 24 hours, the culture medium was substituted for 200 μL RPMI 1640 with 10% human serum and 5×10^3 T cells of the YKIII.8 clone per well. After another 24 hours of culture, supernatant was harvested and IFN-γ production was measured by ELISA.

Construction of Minigenes and Peptide Synthesis. The cDNA clone containing the full-length RPS4Y sequence was used as a template for PCR with a common sense primer 5′-GAAGCTAGCATGGCCCGGGGCCCCAAG-3′ and one of antisense primers containing a TAA stop codon in frame with the main open reading frame: minigene 1-124, 5′-TAAGCGGC-CGCTTAGCACAACTTGTACTTTGCC-3′; minigene 1-134, 5′-TAAGCGGCCGCGGTAGCGGATGGTTCGAGC-3′; minigene 1-150, 5′-TAAGCGGCCGCTTAAATCAATCTGCA-CAGTATCG-3′; minigene 1-164, 5′-TAAGCGGCCGCT-TAGGTGATCACACCAACAACG-3′; minigene 1-197, 5′-TAAGCGGCCGCTTATGCCAATGACAAAAATGTTGG-3′; and full-length RPS4Y, 5′-TAAGCGGCCGCTTAGCCAC-TGCTCTGTTTGG-3′. These PCR products were digested with *Nhe*I and *Not*I and cloned into the pCEP4 vector. Transfection of constructs containing truncated *RPS4Y* genes into 293-EBNA-B7-B*5201 cells and screening with YKIII.8 cells were done as described for the cDNA library screening. Candidate peptides were synthesized by solid-phase peptide synthesis and characterized by mass spectrometry (Pepscan Systems, Lelystad, the Netherlands).

Cloning of the YKIII.8 T-Cell Receptor. Usage of variable regions of TCRα (AV) and TCRβ (BV) chains in YKIII.8 was analyzed by reverse transcription-PCR using forward primers specific for different *AV* and *BV* gene families; an oligonucleotide specific for either Cα or Cβ was used as a reverse primer (18). Full-length YKIII.8 TCR (AV17 ~ AJ44 ~ AC, Genbank accession no. AY834221) was amplified with primers 5′-CCCGTCGACATGGAAAC-TCTCCTGGGAG-3′ and 5′-CCCGCGGCCGCCCTCAGCTG-GACCACAGC-3′ and then cloned into *Bam*HI and *Not*I sites of pMX-mTCRα-IRES-EGFP vector. YKIII.8 TCRβ (BV13 ~ BJ1-6*02 ~ BC1, Genbank accession no. AY834220) was amplified with primers 5′-GGGCCGCGGATGCT-TAGTCCTGACCTGCCTGAC-3′ and 5′-CCCGTCGACTCC-TAACTCCACTTCCAG-3′, blunted with a Klenow fragment (Invitrogen), and cloned into blunt *Bam*HI and *Sal*I sites of pMX-mTCRα-IRES-EGFP vector.

Retroviral Transduction and Immunomagnetic Purification. Retroviral vectors were transfected into the amphotropic Phoenix packaging cell line with the calcium phosphate precipitation method (Qiagen). Viral supernatants were harvested

on the second and third days after transfection. 293-EBNA-B7 cells and fibroblasts were incubated for 24 hours with the 1:3 diluted viral supernatant in the presence of 6 μg/mL polybrene (Sigma-Aldrich, Steinheim, Germany). T cells were converted into PHA blasts 2 days before transduction by culture of donor PBMC in RPMI supplemented with 5% FCS and β-mercaptoethanol in the presence of 1 μg/mL PHA and 300 IU/mL IL-2. PHA blasts, EBV-LCLs, and CD40-activated B cells were transduced in nontreated flasks (Becton Dickinson, Franklin Lakes, NJ) coated with 12.5 μg/mL retronectin (Takara, Otsu, Shiga, Japan). Cells were added at a concentration of 1×10^6 cells/mL in the culture medium supplemented with 300 IU/mL IL-2 for PHA blasts and 10 ng/mL IL-10 and 200 units/mL IL-4 for CD40-activated B cells. Fresh viral supernatant was added on the second day. On the third day, cells were harvested and resuspended in culture medium with addition of corresponding cytokines. Two days after harvesting, the fraction of transduced cells was determined by flow cytometry and $NGFR^+$ cells were purified by the MiniMACS separation method (Miltenyi Biotech, Bergisch Gladbach, Germany) using the anti-NGFR monoclonal antibody 20.4. The diluted antibody (20 μL per 1×10^6 cells) was added to cells that were washed once in PBS supplemented with 1% FCS. After incubation with antibody at 4°C for 15 minutes, cells were washed again, and goat anti-mouse IgG microbeads (Miltenyi Biotech) were added at 20 μL per 10^7 cells followed by incubation for 10 minutes at 4°C. Then, cells were separated over the mass spectrometry MACS column according to the manufacturer's protocol (Miltenyi Biotech) and incubated with phycoerythrin-conjugated goat anti-mouse immunoglobulin to determine the purity of the selected population by fluorescence-activated cell sorting analysis. NGFR-sorted $TCR^{YKIII.8}$-transduced T cells underwent one round of expansion done according to the protocol used for the YKIII.8 T-cell clone culture.

Chromium Release Assay. The chromium release assay was done according to the previously described protocol (19) with minor modifications. Briefly, 2,500 labeled target cells per well were seeded in triplicate in 96-well round-bottomed plates (Costar, Cambridge, MA) in 200 μL RPMI 1640 supplemented with 5% FCS. Thereafter, target cells were incubated for 4 hours at 37°C with T cells at different E:T cell ratios. Supernatants were collected using the Supernatant Harvesting System (Molecular Devices Corp., Sunnyvale, CA) and radioactivity was measured with a Cobra autogamma betaplate reader (Packard, Groningen, the Netherlands).

IFN-γ Production Assay. Target cells (3×10^4) were cocultured with T cells (3×10^4) in 96-well round-bottomed plates in triplicates. After 24 hours, the supernatant was harvested and IFN-γ concentration was measured by the PeliPair human IFN-γ ELISA reagent set (CLB, Amsterdam, the Netherlands) according to the manufacturer's instructions.

Flow Cytometric Determination of Cell Surface Markers. Flow cytometry was done on a Calibur flow cytometer (BD Biosciences, San Jose, CA). Monoclonal antibodies used for the flow cytometry were purchased from BD Biosciences, except 20.4 anti-NGFR antibody (culture supernatant) and goat anti-mouse immunoglobulin phycoerythrin-conjugated antibody (SBA, Birmingham, AL). Data analysis was done using CellQuest software (BD Biosciences).

Blocking Experiments. Blocking experiments were done using the following blocking antibodies kindly provided by Dr. Harry Dolstra (University Hospital Nijmegen, Nijmegen, the Netherlands): anti-CD8-WT82a; anti-CD4-RIV-7; anti-HLA class I-W6/32; anti-HLA class II (anti-HLA-DR/DP)-Q5/13; and anti-HLA-BC-B1.23.3. Antibodies were used at a final concentration of 10 μg/mL and were present throughout the assay.

Western Blot Analysis. Cells were lysed with Laemmli sample buffer and protein quantification was done with Bradford reagent (Bio-Rad, Hercules, CA). Protein extracts (50 μg) were separated on 15% SDS-polyacrylamide gels. The gel was electrophoretically transferred to Hybond-P membrane (Amersham Pharmacia, Freiburg, Germany), blocked in TBS/0.1% Tween containing 5% nonfat dry milk, and incubated with a polyclonal antibody reacting to the female isoform of RPS4 protein at 1:1,000 dilution (20). As an internal control, staining with an anti-actin antibody (Sigma-Aldrich) at 1:10,000 dilution was done concomitantly. Primary antibodies binding to RPS4X and actin were detected by incubation with horseradish peroxidase–conjugated swine anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) diluted 1:1,000. After washing to remove an excess of the secondary antibody, membranes were developed using enhanced chemiluminescence (Amersham Pharmacia).

RESULTS

YKIII.8 Is Specific for a HLA-B*5201-Restricted Minor Histocompatibility Antigen Expressed on Proliferating Lymphocytes. The T-cell line YKIII was generated through repetitive stimulation of T cells of a female donor with CML cells and then later with EBV-LCL of her HLA-identical brother. A limiting dilution culture of YKIII produced several $CD8^+$ T-cell clones that showed specific IFN-γ production toward EBV-LCL of patient (EBV_p) and not donor (EBV_d) origin. The CTL YKIII.8 was selected for further study because of its particular pattern of reactivity toward different subsets of the patient's primary leukocytes. Significant reactivity was observed toward PHA-activated T-cell blasts and to a lesser extent toward CD40-activated B cells (Fig. 1*A*). In contrast, little or no reactivity was observed toward the patient's unfractionated CML cells, normal $CD14^+$ cells, and unstimulated primary T and B cells (Fig. 1*A*). The lytic activity of YKIII.8 followed the same pattern (Fig. 1*B*). This suggested that YKIII.8 recognized a differentially expressed mHag.

YKIII.8 reactivity toward EBV_p was completely blocked by incubation with antibodies against HLA class I, HLA-B and HLA-C molecules, and CD8 (Fig. 1*C*) but not with antibodies against HLA class II or CD4. These results indicated that YKIII.8 reacted toward an antigen presented on either HLA-B or HLA-C and that its recognition required co-engagement of the CD8 coreceptor. To identify the exact restriction element of YKIII.8, we transduced a random selection of 19 EBV-LCL cell lines derived from members of two CEPH families with retroviruses encoding either of the two *HLA-B* alleles expressed by the patient—HLA-B*3508 and HLA-B*5201. None of the mock-transduced EBV-LCLs or EBV-LCLs transduced with B*3508 were recognized by YKIII.8, whereas 10 of 19 EBV-LCLs transduced with B*5201 induced IFN-γ production by YKIII.8. Intriguingly, all recognized EBV-LCLs were derived from male donors, demonstrating that YKIII.8 recognized a H-Y antigen presented on HLA-B*5201.

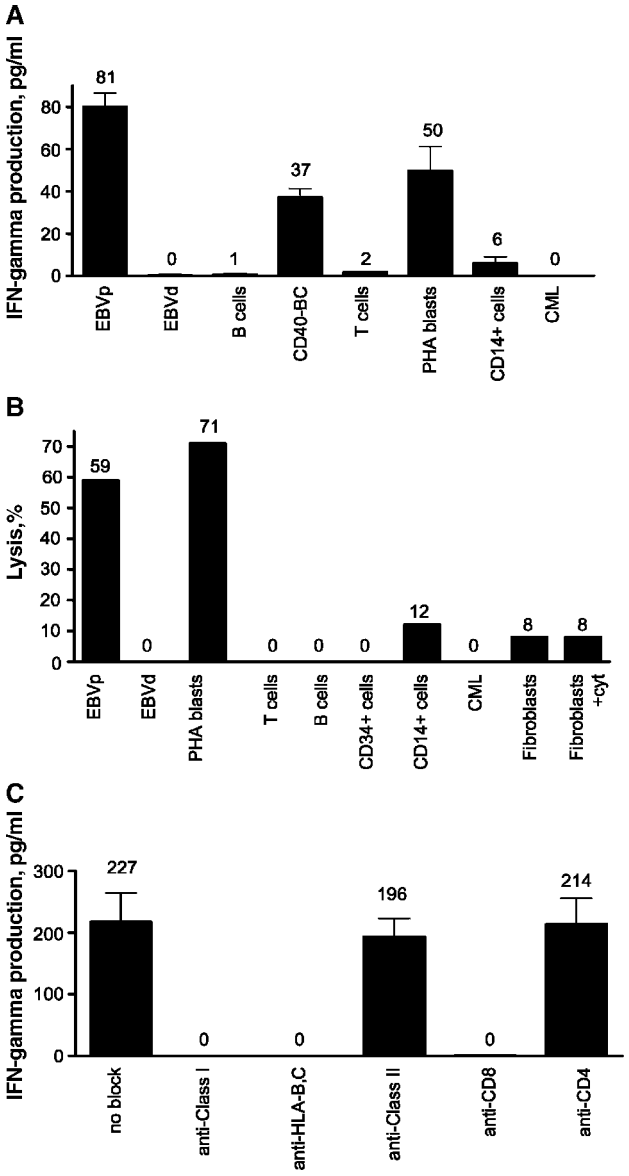

Fig. 1 YKIII.8 reacts toward a mHag expressed mainly on activated lymphocytes and presented on either HLA-B or HLA-C. *A,* IFN-γ production by YKIII.8 in response to stimulation with different cell types for 24 hours was measured by an IFN-γ ELISA. YKIII.8 displayed preferential recognition of activated lymphocytes of patient origin. *B,* YKIII.8 efficiently lysed EBV-LCL and PHA blasts of patient origin in a ^{51}Cr release assay at an E:T of 30:1. Under the same conditions, lytic activity toward immunomagnetically purified nonstimulated patient T and B lymphocytes, $CD14^+$, $CD34^+$, CML cells, and $B*5201^+$ male fibroblasts was much lower. Fibroblast recognition did not increase after pretreatment with 5 ng/mL IFN-γ and 100 units/mL TNF-α for 48 hours (*cyt*). *C,* IFN-γ production by YKIII.8 was completely abolished after incubation of T cells with the CD8 monoclonal antibody and after incubation of EBV_p with HLA class I monoclonal antibody or anti-HLA-B/HLA-C serum.

As shown in Fig. 1*A* and *B*, YKIII.8 responded to stimulation not only with EBV-LCL but also with normal B and T lymphoblasts of the patient. To confirm the ability of YKIII.8 to recognize proliferating male lymphocytes other than EBV-LCLs, we tested YKIII.8 reactivity toward $B*5201^+$ B and T cell blasts of three male individuals. Due to the low frequency of the *B*5201* allele in Caucasians, we had to perform B*5201 transductions of EBV-LCLs, PHA blasts, and CD40-activated B cells of three $B*5201^-$ male donors. The transduction efficiency, measured by fluorescence-activated cell sorting analysis of the *NGFR* marker gene expression, was 18.3 ± 2.9%, 42.7 ± 14.7%, and 7.7 ± 2.9% for EBV-LCL, PHA blasts, and CD40-activated B cells, respectively. A sharp increase of reactivity toward both EBV-LCLs and PHA blasts was observed on transduction with B*5201 (Fig. 2*A*). The increase of reactivity toward CD40-activated B cells was weak possibly due to the low transduction efficiency.

In a separate experiment, the $NGFR^+$ fraction of fibroblasts transduced with B*5201 induced only a weak YKIII.8 response (1% of the IFN-γ production on EBV_p; Fig. 2*B*). To increase the immunogenicity of $B*5201^+$ fibroblasts, they were pretreated with IFN-γ and TNF-α 2 days before the test, which increased HLA class I expression ~5-fold (Fig. 2*C*). A slight increase in the YKIII.8 reactivity was observed as a result, although it made up only a small fraction (5.4%) of the IFN-γ production toward EBV_p (Fig. 2*B*). YKIII.8 cytolytic activity toward fibroblasts was also very low (Fig. 1*B*). Taken together, these data indicated that YKIII.8 reacted toward a B*5201-restricted male-specific mHag that is overexpressed in proliferating lymphocytes.

Identification of RPS4Y as a Male-Specific Antigen Capable of Inducing CTL Responses. To identify the nature of the antigen recognized by YKIII.8, we created a cDNA library from EBV_p, consisting of ~10^6 independent clones with an average size of insert ~1.8 kb and a frequency of recombinants of >90% (data not shown). Screening of 720 pools of 50 individual clones revealed 11 pools, which after transfection into 293-EBNA-B7-B*5201 cells induced IFN-γ production by YKIII.8. Three pools that were the most potent stimulators of YKIII.8 activity were subcloned and five individual clones were isolated from them. Electroporation of EBV_d with these individual plasmids led to their recognition by YKIII.8 (186 pg/mL IFN-γ production versus 5 pg/mL on EBV_d transfected with a mock plasmid versus 884 pg/mL on EBV_p). Sequencing of cDNA inserts showed that all positive clones contained the 931-bp full-length cDNA of the male isoform of RPS4Y (Genbank accession no. NM_001008). This gene encodes a structural component of the 40S ribosomal unit, consisting of 263 amino acids, which is different from its female analogue (Genbank accession no. NM_001007) by 19 amino acids and is expressed in all male tissues. 293-EBNA-B7-B*5201 cells transfected with a pCEP4 vector encoding the female isoform of RPS4 protein were not recognized by YKIII.8 (Fig. 3*A*).

YKIII.8 Recognizes a TIRYPDPVI Nonapeptide of RPS4Y. To identify the epitope of RPS4Y recognized by YKIII.8, we cloned six truncated *RPS4Y* genes, missing different parts of the 3′ sequence. Only minigenes containing a sequence coding for amino acids 150 to 164 induced YKIII.8 activation on their transfection into 293-EBNA-B7-B*5201 cells (Fig. 3*A*). Therefore, the peptide recognized by YKIII.8 is localized fully or partially between amino acids 150 and 164. The male and female isoforms of RPS4 differ in this region at two amino acid positions. Twelve overlapping peptides spanning this region were synthesized (Fig. 3*B*). EBV_d were loaded with these candidate peptides

and tested for their ability to induce IFN-γ production by YKIII.8. EBV_d loaded with the peptide TIRYPDPVI were recognized by YKIII.8, whereas no reactivity was observed toward nontreated EBV_d or EBV_d loaded with other peptides (Fig. 3*C*).

In a separate experiment, the recognition of octamers and decamers, which include the whole or part of the TIRYPDPVI sequence, was tested. Only decamer RTIRYPDPVI induced significant activation of YKIII.8 (Fig. 3*D*). However, it was recognized less efficiently than TIRYPDPVI (Fig. 3*E*). Remarkably, EBV_d loaded with the female counterpart TIRYPDPLI did not induce significant activation of YKIII.8 (Fig. 3*D* and *E*). Interestingly, a minimal TIRYPDPVI concentration of 10^{-7} mol/L was required to induce activation of YKIII.8 (Fig. 3*E* and *F*). In a parallel experiment, a 100 times lower concentration of HA-1 peptide was enough to activate a high-avidity HA-1-specific T-cell clone (21).

RPS4 Proteins Are Overexpressed in Proliferating Normal and Malignant Leukocytes. The level of RPS4 expression in several types of blood cells was studied to analyze whether differential reactivity of YKIII.8 toward various leukocyte subsets correlated with differential levels of RPS4 expression. A Western blotting analysis of lysates of different leukocyte subsets was done with an antibody reacting toward the female isoform of RPS4 (RPS4X). Results of Zinn et al. indicated that RPS4Y is expressed at a fixed ratio of ~ 1:10 to RPS4X (22). To confirm these findings, we analyzed the ratio between RPS4Y and RPS4X mRNA levels by a real-time reverse transcription-PCR with primers specific for either male or female isoforms of RPS4. We found a rather fixed RPS4X/RPS4Y mRNA ratio in a random selection of EBV-LCL, PHA blasts, and homogenized foreskin samples (RPS4X/RPS4Y = 8.63 ± 1.83; n = 8). Therefore, differences in the level of RPS4X expression detected in our experiments are likely to correlate with a differential RPS4Y content and an amount of total RPS4 protein in tested samples. A significant overexpression of RPS4 was found in EBV-LCL, PHA blasts, and CD40-activated B cells of the original patient in comparison with the level of expression in unstimulated T and B cells. Additionally, we studied RPS4 expression in blood cell subsets of three male donors. We found its overexpression in EBV-LCLs of all three donors. In two of three studied donors, a significant overexpression of RPS4 in PHA blasts and CD40-activated B cells was found (Fig. 4*A*). Therefore, elevated expression of RPS4 in lymphoblasts correlated with their recognition by YKIII.8.

The same approach was used to study RPS4 expression in a selection of bone marrow samples from patients suffering from different hematologic malignancies. All of them had >80% malignant cells in the bone marrow. We found an increased level of RPS4 expression in three of three multiple myeloma, three of three acute lymphoblastic leukemia, and two of three acute myelogenous leukemia samples in comparison with pooled normal PBMC of three male donors (Fig. 4*B*). Importantly, RPS4 level in cultured fibroblasts was lower than in EBV-LCL and did not change after 2 days of culture in the presence of IFN-γ and TNF-α (Fig. 4*C*). In normal bone marrow, RPS4 was expressed at the same low level as in normal PBMC (Fig. 4*D*). Furthermore, an overexpression of RPS4 in all tested cancer cell lines was detected (Fig. 4*D* and *E*). Together, these data confirm the association between RPS4 overexpression and high proliferative status.

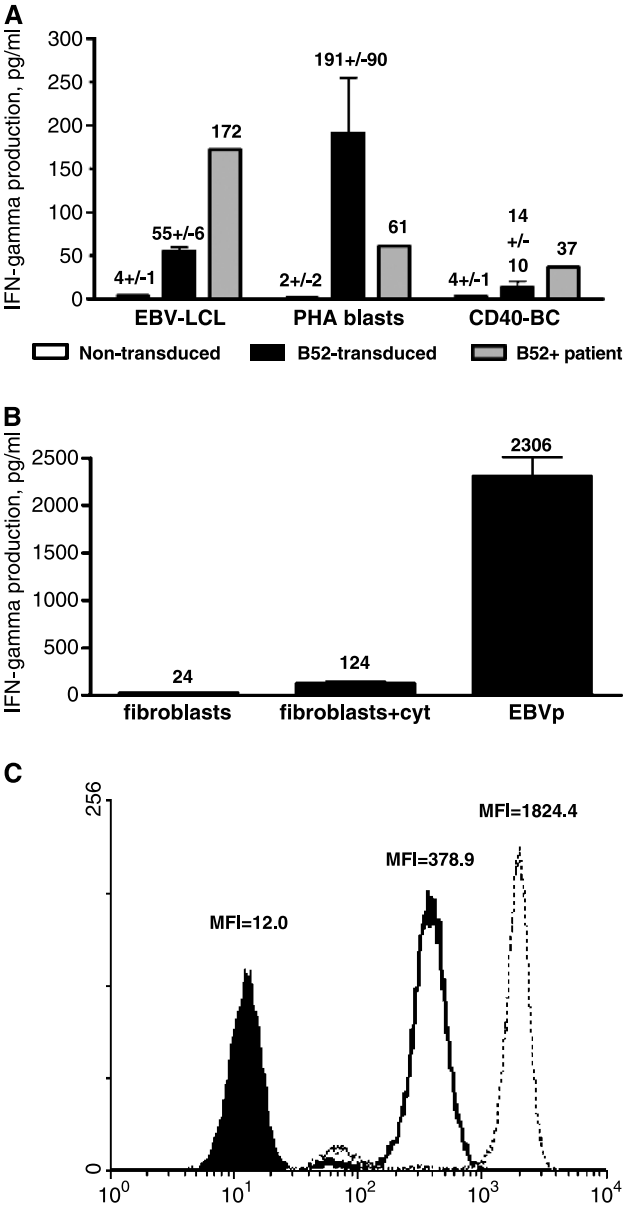

Fig. 2 YKIII.8 recognizes male B*5201-transduced EBV-LCLs, PHA blasts, and CD40-BC but not fibroblasts. *A,* IFN-γ ELISA was used to measure IFN-γ production by YKIII.8 in response to stimulation for 24 hours with male EBV-LCLs, PHA blasts, and CD40-BC transduced with B*5201. Columns, average measurements for three patients; bars, SDs. IFN-γ production in response to stimulation with corresponding cell types of the original $B5201^+$ patient is shown for comparison. PHA blasts were cultured for 5 days in the absence of PHA before the test. Without addition of YKIII.8, $B*5201^+$ patient's PHA blasts produced only 2 pg/mL IFN-γ, nontransduced $B*5201^-$ PHA blasts produced 3 ± 2 pg/mL, and B*5201-transduced PHA blasts produced 18 ± 16 pg/mL. *B,* IFN-γ ELISA was used to measure IFN-γ production by YKIII.8 in response to 24 hours of stimulation with EBV_p, $B*5201^+$ male fibroblasts, and the same fibroblasts pretreated with 5 ng/mL IFN-γ and 100 units/mL TNF-α for 48 hours. *C,* HLA class I expression on fibroblasts was measured by fluorescence-activated cell sorting analysis after staining with a FITC-conjugated antibody. HLA class I expression on nontreated fibroblasts and fibroblasts incubated with 5 ng/mL IFN-γ and 100 units/mL TNF-α for 48 hours is shown by empty contours with continuous and dotted borders, respectively. Filled contour, nonstained fibroblasts. MFI, mean fluorescence intensities.

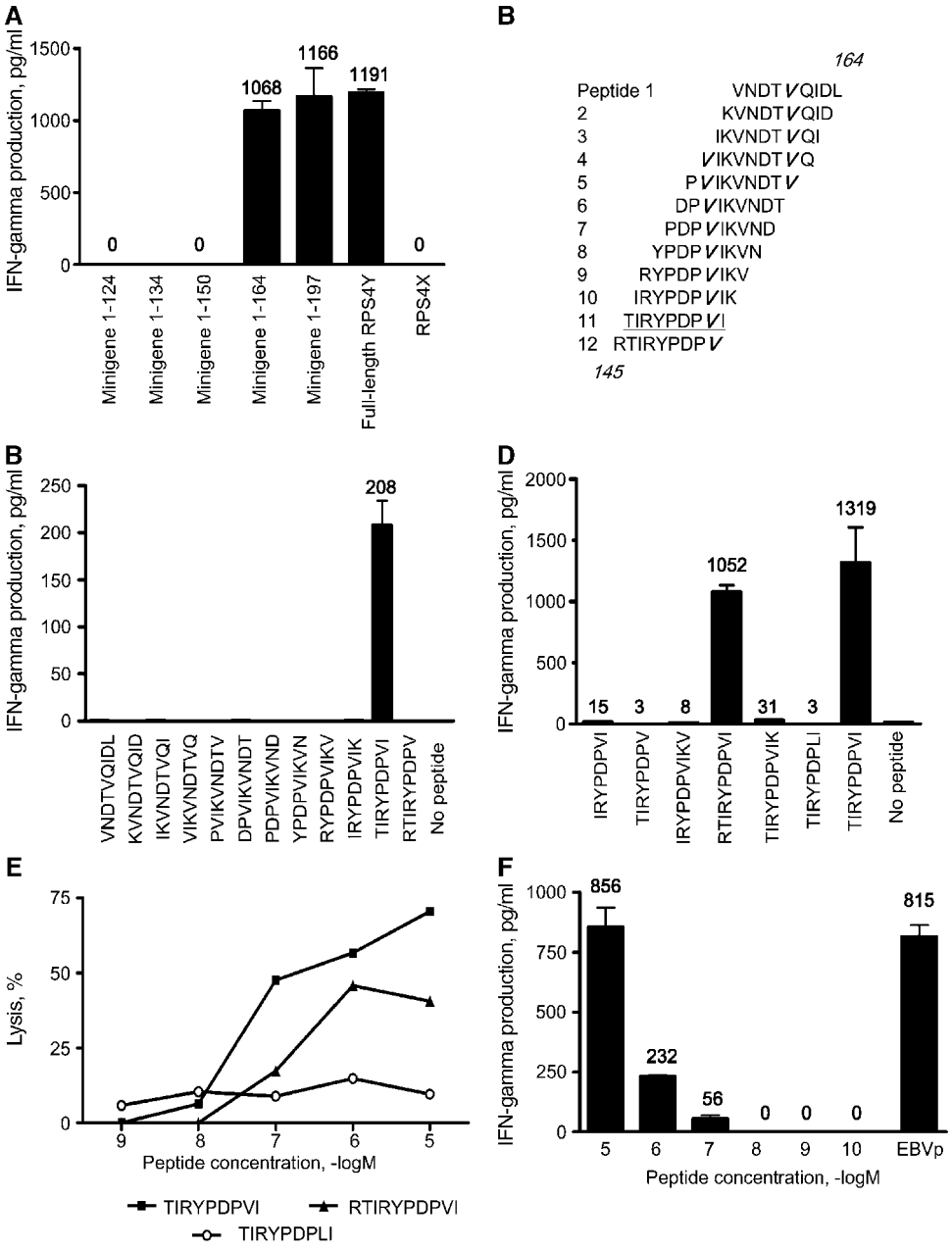

Fig. 3 YKIII.8 recognizes the TIRYPDPVI peptide of RPS4Y. *A,* YKIII.8 did not recognize 293-EBNA-B7-B*5201 cells transfected with minigenes encoding up to 150 NH_2-terminal amino acids of RPS4Y; all minigenes starting with minigenes 1 to 164 (encoding for 164 N-terminal amino acids) included the sequence of the epitope recognized by YKIII.8. *B,* the region of RPS4Y between amino acids 150 and 164 contains two different amino acid residues comparing with RPS4X: $Leu^{153}Val$ and $Ile^{160}Val$ (*bold italics*). Twelve candidate nonapeptides tested in the further experiment. *C,* the peptide TIRYPDPVI (*underlined* on *B*) was the only nonamer found to be recognized by YKIII.8 in an IFN-γ ELISA. *D,* reactivity of YKIII.8 toward EBV_d loaded with octameric and decameric peptides related to TIRYPDPVI and female counterpart TIRYPDPLI was tested in an IFN-γ ELISA. *E,* lytic activity of YKIII.8 toward EBV_d loaded with increasing concentrations of peptides TIRYPDPVI, TIRYPDPLI, and RTIRYPDPLI was tested in a ^{51}Cr release assay at the E:T of 10:1. *F,* IFN-γ production by YKIII.8 in response to stimulation with EBV_d loaded with decreasing concentrations of TIRYPDPVI peptide was tested in an IFN-γ ELISA.

Retroviral Transfer of YKIII.8 T-Cell Receptor into Donor T Cells Preserves the Specificity and Enhances the Amplitude of the Lymphoblast-Specific Response. T cells of the female donor were cotransduced with two retroviral vectors encoding the α and β chains of the YKIII.8 TCR ($TCR^{YKIII.8}$), respectively. Then, TCRα/$NGFR^+$ T cells were immunomagnetically purified to obtain a cell population with 75% cells positive for both α and β chains of $TCR^{YKIII.8}$ (Fig. 5*A* and *B*). Unlike mock-transduced cells, $TCR^{YKIII.8}$-transduced donor T cells displayed a profound reactivity to EBV_p (Fig. 5*C*). The EBV_p-specific reactivity was completely confined to the $CD8^+$ fraction, confirming the necessity of the coreceptor engagement for T-cell activation via $TCR^{YKIII.8}$, which was found in blocking experiments. Remarkably, the amount of IFN-γ produced by $TCR^{YKIII.8}$-transduced T cells was higher than that of YKIII.8. This suggests that the low levels of IFN-γ produced by YKIII.8 are more likely to be caused by the prolonged *in vitro* expansion of this clone than by the insufficient stimulation through the low-affinity receptor. Surprisingly, we found that both the original T-cell clone and T cells transduced $TCR^{YKIII.8}$ reacted to EBV_d loaded with TIRYPDPVI peptide at the same minimal concentration of 10^{-7} mol/L (Fig. 6*A*). Taking into account the inferior expression of $TCR^{YKIII.8}$ on the surface of $TCR^{YKIII.8}$-transduced cells in comparison with the original clone (Fig. 5*B*), an inefficient generation of TIRYPDPVI or binding to HLA-B*5201 rather than low avidity of YKIII.8 is likely to cause a preferential recognition of target cells.

Notably, $TCR^{YKIII.8}$-transduced T cells efficiently lysed EBV_p and PHA blasts of patient origin (Fig. 6*B*). Moreover, Raji cells were also recognized after transduction with the pMX-B*5201-IRES-NGFR retroviral vector and immunomagnetic purification of $NGFR^+$ cells. In contrast, the reactivity toward other hematopoietic cells and, most importantly, fibroblasts was much lower. In conclusion, $TCR^{YKIII.8}$-transduced T cells displayed the same pattern of differential reactivity as YKIII.8.

DISCUSSION

The results of this study show that the TIRYPDPVI nonameric peptide of the *RPS4Y* gene defines a novel HLA-B*5201-restricted H-Y antigen. RPS4Y is the fifth human *H-Y* gene known to encode a CTL epitope. All of these genes [*SMCY*, *UTY*, *DBY*, *DFFRY* (*USP9Y*), and *RPS4Y*] are ubiquitously expressed (23). However, similar to the HLA-B8-restricted T-cell clone recognizing a peptide of the *UTY* gene (15), the RPS4Y-specific T-cell clone YKIII.8 displayed a selective pattern of reactivity. It reacted toward lymphoblasts only, whereas little or no reactivity was found in response to stimulation with unstimulated lymphocytes or nonhematopoietic cells.

The differential reactivity of YKIII.8 may be explained as follows. Firstly, the unusually high concentration of TIRYPDPVI necessary for T-cell activation in the peptide titration experiments indicates either a low affinity of $TCR^{YKIII.8}$ to the complex of B*5201 and TIRYPDPVI or a low affinity of TIRYPDPVI to B*5201. Primary T cells transduced with $TCR^{YKIII.8}$ had a lower expression of $TCR^{YKIII.8}$ on the surface and therefore a lower avidity for the B*5201/RPS4Y complex than the original YKIII.8 clone. Still, YKIII.8 and $TCR^{YKIII.8}$-transduced T cells required the same minimal peptide concentration for activation. This strongly suggests that despite the CD8 dependence of $TCR^{YKIII.8}$-mediated recognition the avidity was not a limiting factor for T-cell activation. More likely, the differential pattern of target cell recognition is defined by a limited number of B*5201/TIRYPDPVI complexes on the cell surface rather than by the avidity of YKIII.8. This may be due to either a low affinity of TIRYPDPVI for B*5201 [despite the presence of valine in the eighth position that can serve as an anchor residue for binding to B*5201 (24)] or inefficient processing of TIRYPDPVI. Although additional experiments are needed to substantiate this point, we suggest that the low affinity of TIRYPDPVI to HLA-B*5201 is crucial for the differential recognition of this epitope. Competition of TIRYPDPVI with high-affinity peptides would lead to the presentation of this epitope only on those cells that overexpress RPS4Y. As was shown for the tumor-associated antigens FGF-5 and MART-1 and the cancer-testis antigen MAGE, susceptibility of tumor cells to lysis by CTL is defined by a certain threshold level of antigen (25–27). The low affinity of TIRYPDPVI to B*5201 makes this threshold high enough to achieve a differential recognition of high and low RPS4Y-expressing cells by $TCR^{YKIII.8}$-positive T cells.

Another factor, which probably contributes to the sensitivity of lymphoblasts to $TCR^{YKIII.8}$-expressing T cells, is that lymphoblasts have up-regulated expression of components of the antigen processing and presentation machinery, which ensures their susceptibility to recognition by RPS4Y-specific CTLs. Processing and presentation of antigens by other cells and especially nonhematopoietic cells are much less efficient.

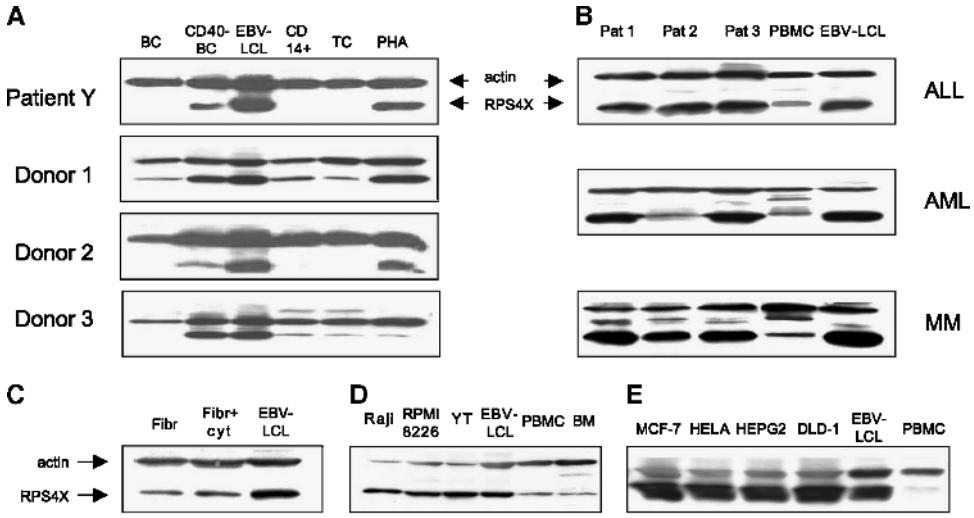

Fig. 4 RPS4 protein is overexpressed in malignant cells and activated normal lymphocytes. RPS4 protein expression was evaluated by Western blot analysis with an anti-RPS4X antibody. Anti-actin antibody was used as an internal control. *A,* lysates of different blood cell subsets from three male donors and the original patient were tested: unstimulated B cells (*BC*), CD40-activated B cells (*CD40-BC*), EBV-LCL, $CD14^+$ cells, unstimulated T cells (*TC*), and PHA blasts. *B,* lysates of bone marrow samples (>80% tumor cells) from nine male patients (three acute lymphoblastic leukemia, three multiple myeloma, and three acute myelogenous leukemia) were analyzed. Lysates of pooled PBMC from healthy male donors and EBV-LCL of the original patient were included for comparison. *C,* lysates of cultured male fibroblasts either treated with IFN-γ and TNF-α (*Fibr + cyt*) or untreated were tested. Lysate of the EBV-LCL of the original patient was included for comparison. *D,* RPS4 was overexpressed in cell lines derived from hematologic malignancies: Raji (Burkitt's lymphoma), RPMI-8226 (multiple myeloma), and YT (T-cell/natural killer cell leukemia). EBV-LCL of the patient, PBMC, and bone marrow from a normal donor are included for comparison. *E,* RPS4 expression was investigated in human cancer cell lines MCF-7 (breast adenocarcinoma), HeLa (cervix carcinoma), Hep-G2 (hepatocellular carcinoma), and DLD-1 (colon adenocarcinoma). EBV-LCL of the patient and PBMC from a normal donor are included for comparison.

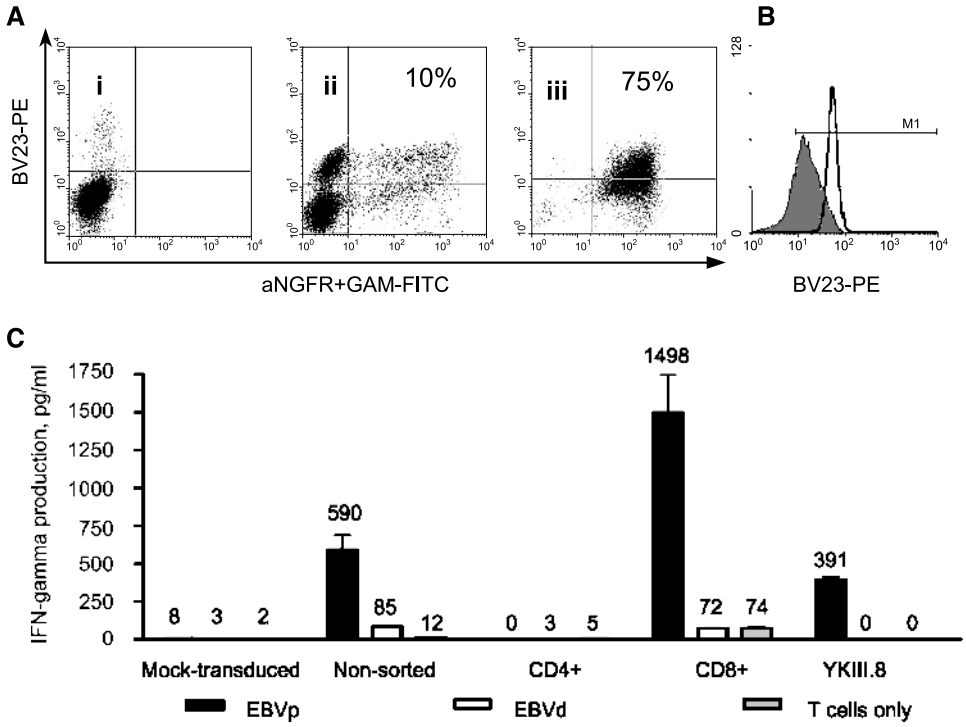

Fig. 5 Specificity of $TCR^{YKIII.8}$ is preserved after transduction of donor T cells. *A,* donor T cells were cotransduced with vectors pMX-$TCR\alpha^{YKIII.8}$-IRES-NGFR and pMX-IRES-$TCR\beta^{YKIII.8}$. Staining with antibodies against TCRVβ23 and NGFR was done on nontransduced (*i*), transduced (*ii*), and transduced and immunomagnetically sorted (*iii*) donor T cells. *B,* $TCR\beta^{YKIII.8}$ expression on TCR-transduced cells was measured by fluorescence-activated cell sorting analysis after staining with phycoerythrin (*PE*)-conjugated BV23-specific antibody. Empty contours, staining of the original YKIII.8 clone. For the mean fluorescence intensity estimation, only $BV23^+$ TCR-transduced cells were gated (marker *M1*) to allow valid comparison with the original clone. Mean fluorescence intensity of YKIII.8 was 60.2 and mean fluorescence intensity of $TCR^{YKIII.8}$ transduced cells was 36.1. *C,* $CD4^+$ and $CD8^+$ fractions of TCR-transduced donor T cells were obtained by immunomagnetic depletion of $CD8^+$ and $CD4^+$ cells, respectively. Reactivity of YKIII.8 and different fractions of TCR-transduced T cells to EBV_p and EBV_d was tested in the IFN-γ ELISA. Columns, average results for triplicates; bars, SD.

Therefore, even if some proliferating and metabolically active somatic cells have a high level of RPS4Y expression, they are unlikely to have a sufficient number of B*5201/TIRYPDPVI complexes on the cell surface for the activation of $TCR^{YKIII.8}$-expressing T cells. Although selective recognition of lymphoblasts may be indicative of immunoproteasome involvement into generation of TIRYPDPVI, it does not seem to be the case because fibroblasts treated with IFN-γ were not recognized by YKIII.8.

Interestingly, there is a clear link between the overexpression of ribosomal proteins and the increased proliferative activity of the cell (28). Several ribosomal proteins have secondary functions as cell proliferation regulators (28). Rapidly proliferating cells, particularly cancer cells, have increased demand in protein biosynthesis; hence, they overexpress several ribosomal proteins that in normal conditions could limit the formation of ribosome particles. For instance, overexpression of the L37 protein was reported in colon carcinomas (29) and transcripts coding for six ribosomal proteins were found among 20 transcripts from cancer cells showing the greatest increase in expression when compared with normal tissue (30). Recently, an association between overexpression of multiple ribosomal proteins with progression of adult T-cell leukemia/lymphoma was found (31). The link between proliferative status and expression of ribosomal proteins was confirmed by our study in which we showed an increased level of RPS4 expression in a selection of hematologic malignancies, EBV-transformed B cells, and activated B and T cells.

Interestingly, a HLA class II–restricted T-helper epitope of RPS4Y was reported recently (32). RPS4Y-specific HLA-DRβ3*0301-restricted T-helper cells were able to mature dendritic cells and promote expansion of mHag-specific HLA class I–restricted $CD8^+$ T cells. This indicates that RPS4Y can induce a comprehensive immune response leading to the formation of immunologic memory.

Tumor-associated antigens, overexpressed in leukemic cells, represent a promising class of potential targets for the immunotherapy of leukemia. Preferential targeting of leukemic cells by CTL specific to tumor-associated antigens, such as WT-1 or hTERT, has been reported (33, 34). Remarkably, generation of CTL specific to a low-affinity hTERT peptide allowed specific recognition of tumor cells and not proliferating healthy cells with lower hTERT levels (35). An expansion of anti-proteinase-3 CTL was shown to correlate with elimination of proteinase-3-overexpressing CML cells in patients, but it did not interfere with normal hematopoiesis (36, 37). However, as a rule, T cells specific to self-antigens are in a state of immunologic tolerance, which makes it difficult to generate CTL responses

targeting tumor-associated antigens. In contrast, H-Y antigens are highly immunogenic for females. Therefore, targeting of H-Y epitopes that despite their ubiquitous expression are preferentially recognized on leukemic cells may represent a possible, although controversial, immunotherapeutic approach. Notably, results of our $TCR^{YKIII.8}$ transfer experiments have shown the feasibility of generation of large amounts of T cells that exert specific lytic activity on lymphoblasts in absence of significant reactivity toward fibroblasts.

Unfortunately, despite the high frequency of HLA-B*5201 in East Asia, where it is shared by 34% Japanese and 27% Chinese individuals (38), the frequency of B*5201 in the Caucasian population is very low (2-6%). Therefore, the YKIII.8 reactivity toward a broader array of malignant and normal tissues awaits further investigation. In addition, identification of RPS4Y-derived peptides able to bind to more frequent *HLA* alleles would allow investigating anti-RPS4Y responses in a larger group of patients. The ability of anti-RPS4Y CTL to prevent leukemic engraftment (39) or eradicate engrafted leukemic cells (40) needs to be tested. Moreover, a correlation between the presence of anti-RPS4Y CTL in the peripheral blood of post-transplant patients, their clinical outcome, and severity of GVHD will provide further clues to the role of anti-RPS4Y CTL in GVL and GVHD.

In conclusion, we have identified a novel H-Y epitope able to elicit CTL responses targeting B and T lymphoblasts. These findings expand the understanding of the nature of GVL responses in female-to-male stem cell transplantation and broaden the array of potential targets for the immunotherapy of hematologic malignancies.

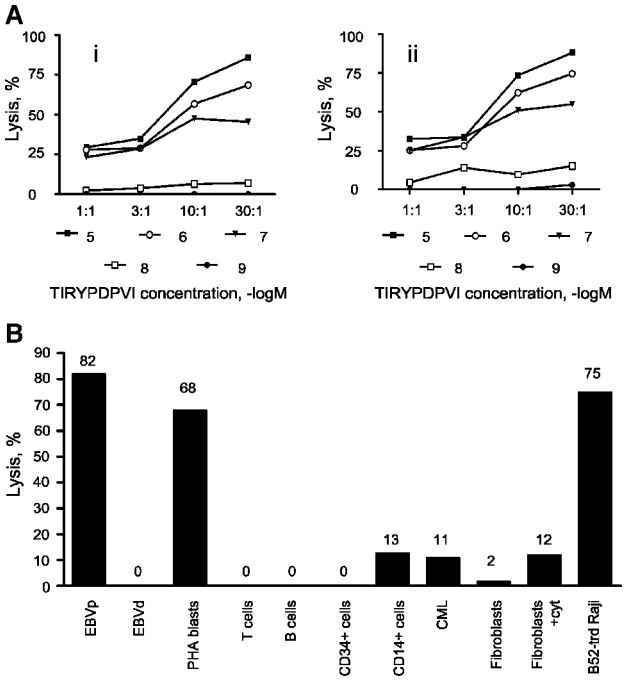

Fig. 6 Sensitivity and differential pattern of recognition by $TCR^{YKIII.8}$-transduced donor T cells. *A,* responses of YKIII.8 (*i*) and TCR-transduced T cells (*ii*) to EBV_d loaded with increasing amounts of TIRYPDPVI were compared in the ^{51}Cr release assay at different E:T ratios. *B,* lytic activity of $TCR^{YKIII.8}$-transduced donor T cells was measured in a ^{51}Cr release assay at an E:T ratio 30:1. Patient's EBV-LCL and PHA blasts were efficiently lysed as well as the $NGFR^+$ fraction of Raji cells transduced with pMX-B*5201-IRES-NGFR. No reactivity toward immunomagnetically purified nonstimulated patient $CD34^+$ cells and T and B lymphocytes was detected. Low reactivity was exerted on $CD14^+$ and CML cells of the patient. Although no significant recognition of $B*5201^+$ male fibroblasts was found, a minor increase of fibroblast recognition was found after pretreatment with 5 ng/mL IFN-γ and 100 units/mL TNF-α for 48 hours (*cyt*).

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