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Differential Splicing of Antigen-Encoding RNA Reduces Endogenous Epitope Presentation That Regulates the Expansion and Cytotoxicity of T Cells[1]

Norbert Kienzle,[2]* Marion Buck,* Sharon L. Silins,* Scott R. Burrows,* Denis J. Moss,* Adam Winterhalter,† Andrew Brooks,† and Rajiv Khanna*

The activation of CTLs is dependent on the recognition of MHC-bound peptide present on the surface of APCs. We give evidence in this study that differential splicing of Ag-encoding RNA can decrease the antigenic dose in APCs and regulate the recall of human memory CTLs. Differential splicing of RNA that encoded an immunodominant HLA-B8-restricted CTL epitope of EBV reduced the functional presentation of this epitope, and consequently the in vitro expansion and activity of CTLs, as measured by MHC/peptide-tetramer staining and cytotoxicity assays. The reduced activity of the stimulated CTLs was not only due to lower numbers of Ag-specific CTLs but, surprisingly, was also characterized by decreased cytotoxicity of the CTLs to target cells presenting limiting amounts of the peptide epitope. As indicated by TCR repertoire analysis, the reduction in CTL activity was not caused by stimulation of distinct populations of TCR clonotypes. This study demonstrates how a common eukaryotic posttranscriptional mechanism of gene regulation can modulate the endogenous presentation of Ag and ultimately contribute to the fine tuning of immunological memory cells, which are important in the fight against pathogens and tumors and in autoimmunity. ***The Journal of Immunology,*** **2000, 165: 1840–1846.**

Cytotoxic T cells are essential components of the adaptive immune system in the fight against intracellular pathogens. Clonal expansion of CTLs and control of their cytotoxic activity are therefore crucial parameters during their life span. A key element in this regulation seems to be the Ag dose presented to CTLs in the context of MHC molecules on the surface of APCs. The numbers of a given MHC-bound peptide complex presented on the cell surface of APCs are thought to be proportional to the activation status of the corresponding T cells that respond to the APCs (for reviews, see Refs. 1–3). The APC's ability to process Ag depends on at least two key features: first, the efficiency of the individual components of the APC machinery, which can be impaired by interactions with proteins expressed by invading pathogens or which may be defective due to intrinsic genetic mutations, as frequently observed in tumor cells. Second, the delivery pathway and the amount of Ag taken up by or expressed within the APC can affect epitope presentation.

In the case of MHC class I-restricted epitopes, molecular mechanisms that regulate the endogenous expression of genes encoding an Ag contribute at a fundamental level to the immunological fate of a CTL epitope. Transcriptional and posttranscriptional regulatory processes such as promoter and codon usage, frame shifts, and aberrant splicing affect the levels of Ag expression and influence the generation or loss of an epitope (for review, see Ref. 4). Indeed, we have recently reported that differential splicing of the EBV-encoded RK-BARF0 transcript generated splice variants whose translated protein isoforms are devoid of an HLA-A2-restricted CTL epitope. This posttranscriptional mechanism reduced the endogenous expression level of this Ag in EBV-infected cells and, consequently, the virus-specific CTL-mediated immune recognition was silenced (5, 6). These data identified a novel and potentially important immune escape mechanism for human pathogens.

Prompted by these findings, we analyzed how the stimulation of virus-specific memory CTLs is affected by the expression of a chimeric form of the RK-BARF0 protein containing an immunodominant HLA-B8-restricted epitope of the EBNA-3 protein of EBV. In this study, we provide evidence that differential splicing of epitope-encoding viral transcripts effectively reduces the levels of epitope presented by the APC, as measured by cytotoxicity assays, and thereby negatively modulates both quantitatively and qualitatively the epitope-specific CTL response.

Materials and Methods

Cell lines and viruses

The lymphoid cell lines (LCLs)[3] KK-B95.8 and LC-B95.8 were established by exogenous transformation of peripheral B cells using EBV strain B95.8, as described recently (7). Cells were propagated biweekly in RPMI 1640 media containing 2 mM glutamine, 60 μg/ml benzylpenicillin, 100 μg/ml streptomycin, and 10% FCS (growth media) at 37°C in a 5% CO_2 atmosphere. The HLA-B8-restricted CTL clones CF34 and LC13 are specific for the FLR epitope of the EBNA-3 of EBV (8). Infection of LCLs with recombinant vaccinia virus expressing the EBV-encoded EBNA-3 gene was outlined previously (9). Generation and maintenance of PHA blasts were performed as described recently (5).

*EBV Unit, The Queensland Institute of Medical Research and University of Queensland Joint Oncology Program, Brisbane, Queensland, Australia; and †Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria, Australia

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[2] Address correspondence and reprint requests to Dr. Norbert Kienzle, Queensland Institute of Medical Research, Post Office, Royal Brisbane Hospital Qld 4029, Australia. E-mail address: norbertK@qimr.edu.au

[3] Abbreviations used in this paper: LCL, lymphoid cell line; CDR, complementarity-determining region; MFI, mean fluorescence intensity.

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Recombinant FLR-APC system

The *Kpn*I-*Bgl*II fragment of vector pSG5 containing a Flag epitope-tagged RK-BARF0 sequence (10) was cloned into the *Kpn*I and *Bam*HI sites of vector pUC19, thereby generating pUC-Flag-RKBARF0. An adapter oligonucleotide (5′-cttaGGATCCT**CGGCCG TTTCTCCGGGGTCGTGCG TATGGGTTA**CGGCCGGATCCattc), which encoded the FLR epitope sequence (bold) and was flanked by both *Bam*HI and *Eag*I sites (underlined), was inserted into the *Bam*HI or *Eag*I sites, respectively, of plasmid pUC-Flag-RKBARF0. The plasmid inserts were then liberated from pUC-Flag-RKBARF0 using *Kpn*I (blunt ended) and *Sal*I and finally cloned into the *Sac*I (blunt-ended)-*Sal*I sites of vector EBO-pLPP (37704; American Type Culture Collection, Manassas, VA). This vector contains an SV40-based expression cassette, the hygromycin resistance gene, and the oriP/EBNA-1 replicon of EBV for episomal replication. The resulting plasmids expressed an N-terminally Flag-tagged, chimeric FLR/RK-BARF0 sequence that contained the FLR epitope either within the differential 5′- and 3′-splice sites of the RK-BARF0 sequence (E-FLR) or upstream of the spliced-out region (B-FLR) (Fig. 1*A*). Sequencing confirmed the integrity of the constructs. LCLs were stably transfected with constructs E-FLR, B-FLR, or the vector EBO-pLPP using conditions described recently (5, 11), and the transfected polyclonal LCLs were maintained in growth media with hygromycin B (Boehringer Mannheim, Indianapolis, IN) at 150 μg/ml.

The differential splicing of the chimeric FLR/RK-BARF0 transcripts in transfected LCLs was analyzed by RT-PCR using primers specific for the N-terminal Flag epitope and the C-terminal end of RK-BARF0. In accordance with our previous findings for the viral RK-BARF0 sequence (6), the use of the 5′- and 3′-splice sites in the constructs of B-FLR and E-FLR produced major spliced isoforms lacking the area illustrated in Fig. 1*A* and only small amounts of full-length transcripts (data not shown). For immunoblot analysis, total cell extracts of transfected LCLs were separated by a 12% SDS-PAGE and transferred to nitrocellulose, and protein expression was analyzed using the anti-Flag M2 mAb (1/300 diluted; Kodak, Rochester, NY; Fig. 1*B*), essentially as described recently (11).

Polyclonal CTL stimulation

A total of 2×10^6 PBMC, separated on Ficoll-Paque from whole blood of healthy HLA-B8 and EBV double-positive human donors, was cocultivated with gamma-irradiated (8000 rad) stimulator APCs in 2 ml of growth media for 8 days. Different ratios of PBMCs to stimulator cells (125:1 to 1000:1) were tested by varying the numbers of APCs. For long-term restimulation, APCs and rIL-2 (20 U/ml) were added once per week after the initial stimulation.

Cytotoxicity assay

CTLs were tested in duplicate for cytotoxicity in a standard 5-h ^{51}Cr release assay (7), and the procedure was outlined in detail recently (5). For exogenous peptide sensitizing, target cells (PHA blasts or LCLs) were preincubated with synthetic peptides (Chiron Mimotopes, Melbourne, Australia) under saturating (5–10 μg/ml) or limited ($\leq$1 μg/ml) peptide concentrations, and the uncoated peptide was washed off. The HLA restriction of the stimulated polyclonal CTLs was confirmed by anti-HLA class I (W6/32, HB-95; American Type Culture Collection) and anti-CD8 (8014; American Type Culture Collection) Ab inhibition experiments, and by the use of non-HLA-B8-matched PHA blasts (data not shown).

Generation of FLR-tetramers

Tetrameric HLA-B8/FLR peptide complexes were prepared essentially as described by Altman et al. (12). Briefly, recombinant HLA-B8 and human β_2-microglobulin, produced in *Escherichia coli*, were solubilized in urea and injected together with the synthetic FLRGRAYGL peptide into a refolding buffer consisting of 100 mM Tris, pH 8, 400 mM arginine, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione. Refolded complexes were purified by anion-exchange chromatography using DE52 resin (Whatman, Tewksbury, MA), followed by gel filtration through a Superdex 75 column (Amersham Pharmacia Biotech, Piscataway, NJ). The refolded HLA-B8/FLRGRAYGL peptide complexes were biotinylated by incubation for 16 h at 30°C with the BirA enyzme (Avidity, Boulder, CO). Tetrameric HLA/peptide complexes were produced by the stepwise addition of extravidin-conjugated PE (Sigma, St. Louis, MO) to achieve a 1:4 molar ratio (extravidin-PE:biotinylated class I).

Cell staining and FACS analysis

Stimulated polyclonal T cells ($2–5 \times 10^5$) were incubated with the FLR-tetramer (1/100 diluted) and TriColor anti-human CD8 (1/100 diluted; Caltag, Burlingame, CA) in 100 μl of growth medium at standard 4°C for 50 min and then washed twice in PBS containing 1% FCS. To test the temperature specificity of the tetramer binding (13), cells were incubated with the FLR-tetramer at 37°C for 30 min, washed, then incubated with TriColor anti-human CD8 at 4°C for 30 min, and finally washed twice. Stained cells were analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA) using CellQuest software. Logical gating on forward and side scatter selected for activated T cells with minimal autofluorescence and cell death. For cell sorting, T cells were stained with the FLR-tetramer at 4°C and sorted with propidium iodide (5 μg/ml) at 4°C using a FACSvantage (Becton Dickinson). The specificity of the FLR-tetramer binding was confirmed in experiments using HLA-unmatched CTL clones and PBMCs (data not shown).

TCR-Vβ amplification and repertoire diversity analysis

FLR-tetramer-sorted T cells or unsorted PBMCs ($1–1.5 \times 10^5$) were used for RNA extraction (Total RNA Isolation Reagent; Advanced Biotechnologies, London, U.K.). First strand cDNA was synthesized using an antisense TCR-Cβ primer (Cb1), as described previously (14). TCR-β rearranged sequences were amplified with each of 26 different 5′ Vβ-specific primers (Vβ1–5.1, Vβ5.2–25) and a 3′ TCR-Cβ constant primer (15). Amplifications were performed in 25-μl reactions using 0.5 μl cDNA, 5 pmol of each primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (Ampli-*Taq* Gold), and a GeneAmp PCR 9600 system (Perkin-Elmer Cetus, Norfolk, CT). The PCR conditions consisted of an initial denaturation at 95°C for 9 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 5 min. Four percent of this material was then used for a subsequent round of PCR under identical conditions, but for only 10 cycles.

The technique of CDR3 length determination and distribution to analyze TCR repertoire diversity is based on the methodology described previously (16). TCR-Vβ PCR products were labeled with a nested 3′-FAM fluorophore-labeled primer specific for the TCR-Cβ gene (CβP*: 5′-FAM-TTCTGATGGCTCAAACAC-3′; Research Genetics, Huntsville, AL) in a PCR runoff reaction. PCR conditions were identical with those described above, except that 8% of TCR-Vβ product was used as a template for seven cycles of elongation (runoff) and a 5-min final extension at 72°C. The fluorescent PCR runoff products were heat denatured at 95°C for 5 min and were separated on a 6% acrylamide gel together with size standards (GENESCAN-1000 ROX; Applied Biosystems, Brisbane, Australia) on an Applied Biosystems 373A DNA sequencer. Data were processed using the Genescan Analysis 2.1 Software (Applied Biosystems), which records the fluorescence intensity in each peak. The CDR3 length, defined by Chothia et al. (17), is deduced from the fragment size.

Results and Discussion

Differential splicing reduces the presentation and CTL-mediated recognition of an immunodominant epitope

One of the most intriguing features of T cell function is its adaptability to environmental differences, i.e., how changes in Ag levels regulate the activity and diversity of the T cell repertoire. Prompted by our recent findings that differential splicing in APCs can bypass virus-specific CTL recognition (6), we investigated how loss of Ag, induced by differential splicing of Ag-encoding RNA, can influence the recall of human memory CTLs. To this end, a recombinant APC system was designed that, in a splicing-dependent manner, endogenously expressed the immunodominant HLA-B8-restricted epitope FLRGRAYGL (referred to as FLR) of the EBNA-3 protein of EBV in stably transfected LCLs (outlined in detail in *Materials and Methods*). Using Flag Ab epitope tagging, the expression of the FLR-containing protein isoforms was monitored by immunoblot analysis (Fig. 1, *A* and *B*). Construct E-FLR, which contained the FLR epitope within the spliced-out region, generated dominant truncated 16–20-kDa isoforms lacking the FLR epitope and expressed only small amounts (8-fold less, as determined by densitometric analysis) of the 35-kDa full-length, FLR-encoding protein. In contrast, construct B-FLR, containing the FLR sequence upstream of the spliced-out region, expressed the FLR epitope in all the different isoforms independent of their splice status.

The LCL transfectants were analyzed in a cytotoxicity assay at different E:T ratios using a FLR-specific CTL clone (Fig. 1*C*). As

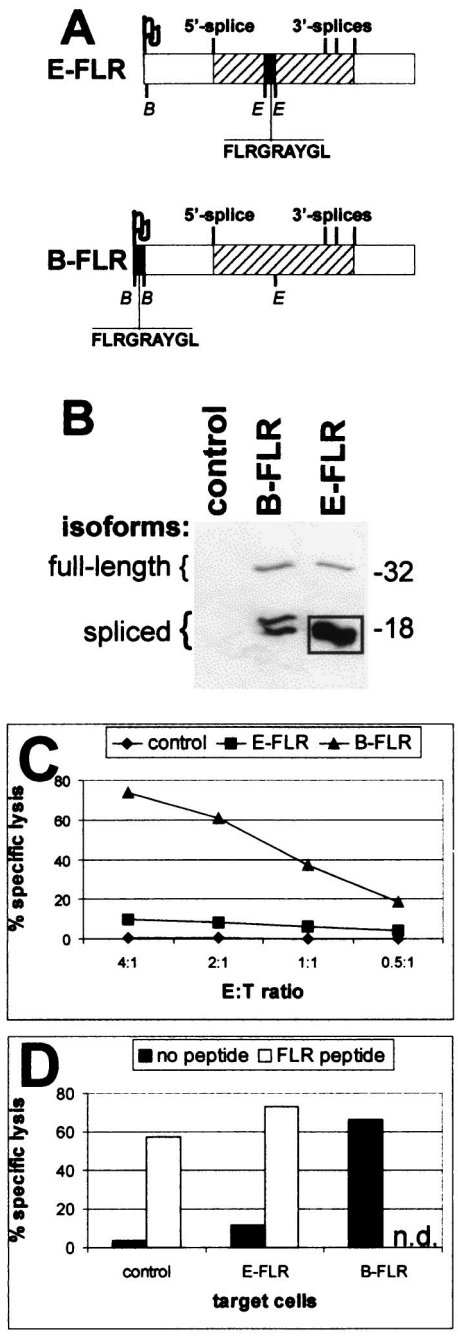

FIGURE 1. Differential splicing reduces the functional presentation of the immunodominant CTL epitope FLR. *A*, Schematic diagram of the N-terminally Flag-tagged (flag) gene constructs that encode the CTL epitope (■) either within (E-FLR) or outside (B-FLR) the spliced-out region (▨) defined by a single 5′-splice and three different 3′-splice sites. Relevant restriction sites for cloning (B, *Bam*HI; E, *Eag*I) and the zoomed-in FLR epitope sequence are illustrated. *B*, Total cell extracts of the LCL LC-B95.8 stably expressing a control vector or constructs B-FLR or E-FLR were analyzed by immunoblot using an anti-Flag mAb. Molecular mass markers (kDa) and the positions of the protein isoforms are indicated. The truncated E-FLR isoforms lacking the FLR epitope are boxed. *C* and *D*, LC-B95.8 cells stably transfected with constructs E-FLR or B-FLR and control cells, either parental or expressing a control vector, were analyzed in a standard ^{51}Cr release assay using the FLR-specific CTL clone CF34 at different E:T ratios. *D*, Target cells, either untreated or coated with the peptide FLR, were assayed with CTL clone CF34 at a 4:1 E:T ratio. n.d., Not done.

the LCLs were immortalized with the EBV strain B95.8, containing a mutation in the last position of its FLR-epitope sequence, the control cells (either parental or transfected with the empty expression vector) showed no significant lysis by the CTL. APCs expressing the B-FLR construct were strongly recognized by the CTL, in contrast to the E-FLR APCs of which recognition was significantly reduced. There was no lack of HLA-B8 cell surface expression in the control or E-FLR-containing APCs as exogenous loading of the FLR peptide onto these target cells restored killing to similar levels seen from the peptide-uncoated B-FLR-expressing APCs (Fig. 1*D*). Equivalent results were obtained with another transfected HLA-B8-positive LCL (KK-B95.8) and with four different FLR-specific CTLs (DD1, WY6, JL24, LC13), which originated from unrelated HLA-B8-positive individuals, showing that differential splicing reduced the FLR Ag expression and subsequent CTL-mediated lysis in APCs up to 9-fold (data not shown).

Reduction in endogenous FLR Ag expression reduces the expansion of stimulated FLR-specific memory CTLs

The next objective was to investigate how differential splicing affected the in vitro recall of memory CTLs from PBMCs of healthy EBV-positive individuals. The experimental protocol assessed 1) the efficacy of APCs that expressed the FLR Ag within the E-FLR or B-FLR constructs, and 2) various ratios of PBMCs to stimulator cells. PBMCs from two unrelated EBV and HLA-B8 double-positive donors (LC and KK) were stimulated once with different numbers of autologous APCs for 1 wk. The activated polyclonal CTLs were then assayed by FACS using an anti-CD8 mAb and a soluble tetrameric complex of FLR peptide-bound HLA-B8 protein (FLR-tetramer). Since a recent publication claimed that the specificity of peptide-tetramer staining can be temperature dependent (13), staining was performed in parallel at 37°C and the standard 4°C. Clearly independent of the staining temperature, there were significantly fewer FLR$^+$ CTLs present in the cultures stimulated with reduced levels of Ag (Fig. 2*A*). After calculating the proportions of FLR$^+$ cells in the total population of CD8$^+$ cells, there was 3.5 (at 37°C)- to 4-fold less (at 4°C) percentage of FLR$^+$/CD8$^+$ CTLs present after stimulation with the APCs expressing the E-FLR construct when compared with B-FLR. No significant differences in the total numbers of CD8$^+$ cells nor in the mean fluorescence intensity (MFI) of the tetramer and CD8 staining were observed comparing the different stimulated PBMC cultures at one given temperature. However, in accordance with Whelan and colleagues (13), incubation at 37°C increased the MFI of staining with the FLR-tetramer complex, presumably due to tetramer internalization. Importantly, no FLR$^+$ CTLs were detected in the B95.8 control cultures, indicating that the expansion of FLR-specific CTLs was indeed due to the recombinant expression of the FLR Ag (encoded by the E-FLR and B-FLR constructs) and not due to the expression of the mutant FLR sequence of the parental B95.8 virus strain. Fig. 2*B* illustrates that independent of the PBMC to stimulator ratio (125:1 to 1000:1) used, the E-FLR-containing APCs, whose FLR expression was reduced due to differential splicing, stimulated consistently fewer percentages of FLR$^+$/CD8$^+$ T cells when compared with B-FLR-expressing APCs. Similar data were obtained from stimulation experiments using PBMCs of donor LC (data not shown).

In parallel, the functional activity of the stimulated polyclonal CTLs was assessed in a standard ^{51}Cr release assay using PHA blasts that were exogenously coated with saturating amounts of FLR peptide. In addition, another HLA-B8-restricted epitope of EBV, RAKFKQLL (referred to as RAK), was used as an internal control as all the stimulator cells expressed the RAK Ag from the parental virus, independent of the E-FLR or B-FLR expression

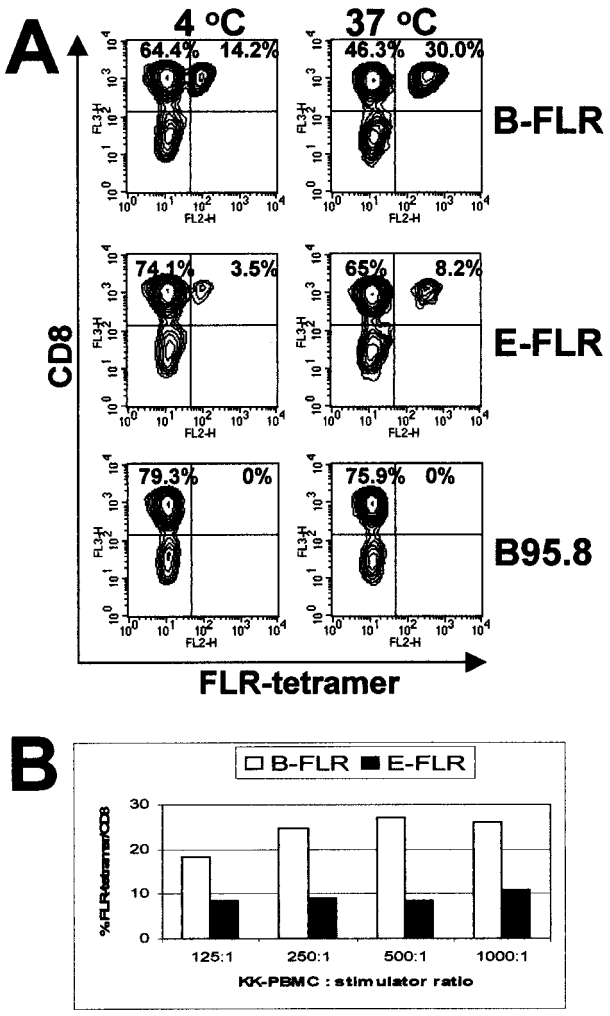

FIGURE 2. Counting FLR-specific CTLs by tetramer staining. PBMCs of the EBV/HLA-B8 double-positive donor KK were stimulated once with autologous APCs expressing the B-FLR or E-FLR construct or with the parental B95.8 LCL at different PBMC to stimulator ratios. *A*, FACS analysis of polyclonal CTLs, stimulated at a PBMC to stimulator ratio of 250:1, using an anti-CD8 Ab and the FLR-tetramer. Tetramer staining was performed at 37°C or standard 4°C, as indicated on the *top*. The percentages of stained cells are given for the *upper left* and *upper right quadrants* of the contour plot. *B*, Summary of the percentage of $FLR^+/CD8^+$ cells after standard FACS analysis of stimulated polyclonal CTLs. The bar legend illustrates the type of stimulator APC used, and the *x*-axis shows the different PBMC to stimulator ratios. The *y*-axis represents the percentage of FLR-specific CTLs (*upper right quadrant*) in the total pool of $CD8^+$ CTLs (*upper left* and *upper right quadrants*).

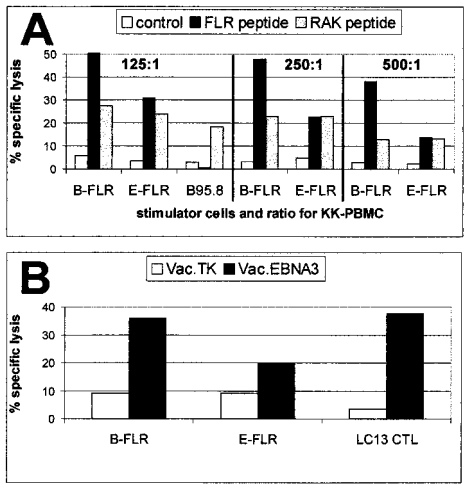

FIGURE 3. Stimulated FLR-specific CTLs recognize exogenously and endogenously presented Ag in a standard ^{51}Cr release assay. *A*, The stimulated polyclonal CTLs generated in Fig. 2*B* were tested at an E:T ratio of 15:1 using HLA-matched PHA blasts that were either untreated (control) or coated with saturating amounts of peptides FLR or RAK. The three different PBMC to stimulator cell ratios analyzed are given at the *top*, and the *x*-axis shows the type of stimulator APC. *B*, Polyclonal CTLs, generated in Fig. 2*B* with a PBMC to stimulator ratio of 500:1, and the FLR-specific CTL clone LC13 were used as effector cells at an E:T ratio of 15:1 and 4:1, respectively. For target cells, autologous B95.8 virus-transformed LCLs were infected with a recombinant EBNA-3 vaccinia virus (Vac. EBNA3) or with control vaccinia virus (Vac. TK).

constructs. Within each set of FLR-expressing APCs (containing the B-FLR or E-FLR construct) and ratio of stimulator cells, the stimulated polyclonal CTLs exhibited similar reactivity against the RAK Ag (Fig. 3*A*). This indicated that there was no difference in the general stimulation capacity of these APCs. In contrast, the reactivity toward the FLR peptide was significantly reduced in the CTL cultures stimulated with the E-FLR compared with the B-FLR-expressing cells. Importantly, this hierarchy of CTL reactivity was preserved for all the different PBMC to stimulator ratios and the two donors tested (data not shown for donor LC). Stimulation with the control B95.8 cells generated no FLR-specific CTLs. The FLR-mediated cytotoxicity was HLA-B8 restricted, as the stimulated CTL cultures did not recognize FLR peptides coated onto HLA-unmatched PHA blasts (data not shown). Thus, these data correlated well with the results from the FLR-tetramer staining, suggesting that the counted FLR-positive CTLs indeed included functional effector cells.

An important question was whether the differentially stimulated FLR-specific CTLs could recognize endogenously presented Ag. Therefore, the cytotoxicity of the T cells was tested in a standard ^{51}Cr release assay using autologous LCLs overexpressing the EBNA-3 gene via a recombinant vaccinia virus. As demonstrated in Fig. 3*B*, both stimulated FLR-specific CTL populations significantly reacted with target cells infected with an EBNA-3 vaccinia virus when compared with vaccinia control virus-infected cells. Importantly, the E-FLR stimulator cells had generated less CTL reactivity toward the target cells expressing the EBNA-3 protein, in contrast to the B-FLR stimulators that had generated CTL reactivity as high as the FLR-specific CTL clone LC13. These data clearly indicated that, independent of the type of stimulator APC used, the expanded FLR-specific CTLs could recognize Ag that was endogenously presented.

We also tested whether prolonged incubation and restimulation of the CTLs could overcome the reduced FLR expression levels in the APCs containing the E-FLR constructs. To this end, PBMCs of donor KK were stimulated for 3 wk with B-FLR- or E-FLR-expressing APCs and then assayed in a standard cytotoxicity and FLR-tetramer staining assay. Surprisingly, restimulation, even in the presence of IL-2, did not equalize the significant differences in numbers of FLR-specific T cell between the two polyclonal CTL cultures, and the hierarchy of the FLR-mediated killing activity was not changed between the first and third stimulation (data not shown). Taken together, these data indicated that the expansion of memory CTLs was dependent on the antigenic dose expressed on the stimulator APC and that moderate differences in Ag expression, caused by differential splicing, effectively regulated the number and activity of stimulated memory cells.

Stimulation with reduced amounts of FLR-Ag results in memory CTLs that are less reactive at low peptide concentration

To study the activity of the stimulated CTLs in more detail, cytotoxicity tests were performed under limited FLR peptide conditions. Independent of the peptide dilution, the reactivity of the CTLs stimulated with the B-FLR-expressing APCs was stronger (up to 3.5-fold) as compared with the E-FLR-stimulated CTLs. This was expected, since the percentage of $FLR^+/CD8^+$ CTLs was 4-fold higher (18.1% vs 4.5%) in the B-FLR population, as counted by FLR-tetramer staining (Fig. 4*A*). To overcome these differences, the E:T ratios were adjusted in the killer assay: the effector cells were diluted (2-fold) in the case of B-FLR and concentrated (3-fold) in the case of E-FLR, resulting now in 1.5-fold (13.5% vs 9.1%) more FLR-specific CTLs in the E-FLR as compared with the B-FLR effector populations. Under the higher concentrations of the diluted FLR peptide, this overcompensation increased the cytotoxicity of the E-FLR CTLs to similar levels, as seen from the B-FLR CTLs (Fig. 4*B*). But surprisingly, at the lowest peptide dilution (0.11 μg/ml), the E-FLR CTL population reacted 2-fold weaker as the B-FLR CTLs. This significant difference, seen at this peptide concentration, was confirmed in two additional experiments showing 2- to 4-fold lower FLR-specific CTL reactivity in the E-FLR, as compared with the B-FLR, CTL population after adjustment of the E:T ratios to similar numbers of FLR-specific CTLs. As an internal control, the recognition of target cells coated with the RAK peptide was tested in a cytotoxicity assay in parallel. As expected, there was a significant higher reaction with the effector cultures stimulated with E-FLR than with B-FLR when using the adjusted, but not the unmodified, E:T ratios (data not shown). This indicated that there was no general immune exhaustion or inhibition in the CTL population stimulated by the E-FLR APCs and that the reduced CTL sensitivity was specific for the FLR epitope.

To determine whether distinct TCR epitope affinities were responsible for the different FLR-CTL sensitivities, the dissociation kinetics of the FLR-tetramer was analyzed using a recently described approach to measure the relative affinity of the TCR for MHC-bound peptide by FACS (18). Over 5-h incubation at 15°C, there was no difference in the off rates of the FLR-tetramer between the two differentially stimulated CTL cultures, either measured as loss of MFI or percentage of $FLR^+/CD8^+$ CTLs. In line with these results, there were no MFI differences between the two different CTL populations regarding the staining with FLR-tetramer and CD8 (data not shown). Taken together, these findings indicated that different TCR-MHC/peptide affinities were not the cause of the reduced cytotoxicity of CTLs, when stimulated with less Ag, but rather suggested that other architectural components of the immunological synapse might have been involved.

FLR-specific CTLs with similar TCR-Vβ repertoires are selected during stimulation with E-FLR- or B-FLR-expressing APCs

As differential splicing in APCs effectively influenced both the quality and quantity of memory CTL recalled, we investigated next whether these moderate differences in the antigenic dose selected distinct populations of T cell clonotypes during stimulation of PBMCs. Therefore, TCR-Vβ repertoire analysis was performed on FLR-tetramer-sorted cells concurrently with the functional studies shown in Fig. 4*A*. Surprisingly, the two functionally different populations displayed the same Vβ usage with near identical CDR3 length repertoire profiles (Fig. 5). Both FLR-specific populations

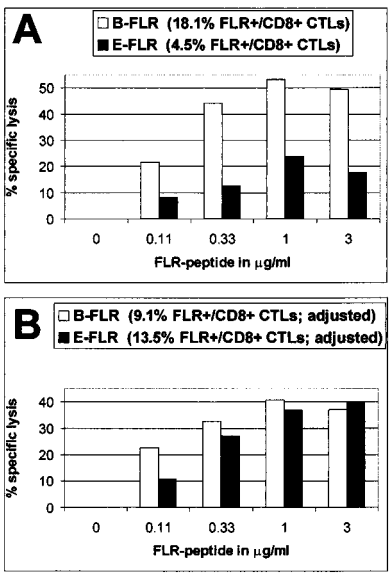

FIGURE 4. Differential splicing of Ag reduces the FLR peptide sensitivity of the expanded CTLs. PBMCs of donor KK were stimulated with APCs expressing the B-FLR or E-FLR constructs at a PBMC to stimulator cell ratio of 250:1. Autologous PHA blasts coated with serial dilutions of the FLR peptide were used as targets in a standard ^{51}Cr release assay. FLR-tetramer staining revealed the numbers of FLR-specific CTLs and the percentage of $FLR^+/CD8^+$ CTL effector cells, either unmodified or adjusted by different E:T ratios, are given in the bar legend. *A*, Both the E-FLR- and B-FLR-stimulated effector cultures were used at an E:T ratio of 12:1. *B*, The E:T ratios were adjusted to 6:1 (B-FLR) and 36:1 (E-FLR), respectively.

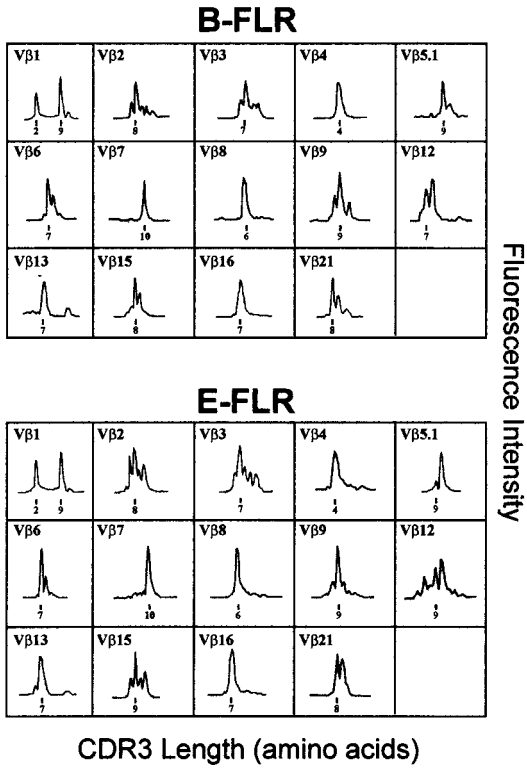

FIGURE 5. TCR-Vβ repertoire analysis of stimulated FLR-specific CTLs. PBMCs of donor KK stimulated with B-FLR- or E-FLR-expressing APCs (generated in Fig. 4*A*) were FLR-tetramer sorted and their TCR usage was analyzed. Profiles are displayed of fluorescence intensity (arbitrary units) as a function of CDR3 size (amino acids) for the different Vβ families. Consecutive CDR3 peaks are spaced 3 nt or 1 aa apart.

used a diverse range of 14 of the known 25 Vβ families (Vβ 1, 2, 3, 4, 5.1, 6, 7, 8, 9, 12, 13, 15, 16, 21), and within each family the CDR3 length distribution was limited to single or few peaks. These results were confirmed in a second independent experiment (data not shown). This shared TCR profile indicated that the same complex mixtures of clonotypes were present in the two different stimulated CTL populations (despite their differences in FLR sensitivity; Fig. 4) and placed emphasis on the importance of the level of Ag expression for recognition by established CTLs, rather than in the induction of the response.

The broad Vβ spectrum selected during stimulation of CTLs of donor KK (HLA-B8/B44.03$^+$) was in line with our previous results showing that EBV- and B8/B44.03-positive individuals do not have a focused FLR-specific TCR repertoire (19). In contrast, the repertoire profile obtained from unstimulated PBMCs of this donor was positive for all the 25 Vβ families and Gaussian in CDR3 length distribution (data not shown), reflecting the normal peripheral blood repertoire of healthy EBV$^+$ donors (20). Thus, CTL populations sharing the same Ag specificity and similar TCR profiles have significantly different cytotoxic capabilities that can be fine tuned by moderate differences in Ag dose.

Implications of differential splicing for APC and CTL function

The plasticity of the T cell repertoire during selection is well established and shows an astonishing malleability toward strong differences in the antigenic dose presented by APCs. For example, stimulation studies with murine cells exogenously coated with peptides showed that 100-1000-fold differences in peptide concentrations dramatically changed the activity of memory CTL lines. Long-term, repeated stimulation with low peptide doses selected high reactive CTLs, as measured by cytokine release, cytotoxicity, and TCR affinity assays, in contrast to high peptide doses that resulted in low reactive CTL lines and clones (21–24). Importantly, when investigated in those studies, the increase in CTL activity was always paralleled by a highly restricted TCR-Vβ usage, indicating that stimulation with low peptide concentrations selected for high avidity TCR clonotypes (23, 24). In contrast, the present study shows that moderate reduction (up to 9-fold) of intracellular Ag, which was endogenously processed and presented on the surface of APC, neither changed the TCR repertoire nor increased the activity of CTLs, but rather selected for less reactive CTLs in the polyclonal cultures. Thus, the observed differences in T cell stimulation by the different experimental systems used highlight the adaptability of T cells for responding to dynamic environments.

Differential splicing is a gene regulatory mechanism used, for example, by viruses generating, from one precursor RNA, different transcripts and subsequent protein isoforms that can vary in quantity and function, and this mechanism can be regulated at the cell and tissue-specific level (for reviews, see Refs. 25 and 26). Our data indicate that differential splicing of Ag-encoding RNA had created limiting conditions for Ag that could shape the expansion and activity of a heterogenous CTL population. The quantity and activity of stimulated memory CTLs could be maintained over time, suggesting that differential splicing of Ag-encoding RNA is an effective regulatory mechanism for the long-term exposure to epitopes expressed, for example, in persistent infections, tumors, and autoimmunity. Thus, differential splicing provides a physiological way to create Ag windows or gradients that can regulate T cell function. Indeed, two recent studies highlighted the biological significance of differential splicing in autoimmunity using the model of experimental autoimmune encephalomyelitis (27, 28). These authors showed that in the thymus, differential splicing led to the removal of an immunodominant epitope encoded in an autoantigen that consequently resulted in the lack of tolerance against this epitope.

The APC model used in this study is based on a recombinant viral FLR/RK-BARF0 gene construct whose individual sequences (RK-BARF0 and the FLR-encoding EBNA-3) are naturally spliced in EBV-infected cells. RK-BARF0 belongs to a family of multispliced transcripts from which a CTL and a B cell Ab epitope is effectively removed by differential splicing (6, 29) .The FLR CTL epitope is located in the EBNA-3 gene that, like the EBNA-4 and EBNA-6 genes, consists of two exons and one intron. We have recently reported that the majority of mRNAs encoding for EBNA-3, EBNA-4, and EBNA-6 retained their introns, and that this form of differential splicing could influence EBNA-3 protein expression (11). It is therefore tempting to speculate that during persistent EBV infection, differential splicing might regulate the immunogenicity of the EBNA-3, EBNA-4, and EBNA-6 proteins that contain ~50% of all the EBV CTL epitopes (reviewed in Ref. 30). On the other hand, differential splicing cannot only reduce Ag, but also generate new CTL epitopes, with some of them potentially useful for tumor or pathogen immunotherapy (4). In combination with, for example, the usage of nonclassical start codons (31) or cryptic promoters (32), these molecular mechanisms can create novel Ag-coding reading frames that multiply the number of potential CTL epitopes for one given gene sequence. In summary, differential splicing is a versatile eukaryotic regulator of gene expression that can modulate, both positively and negatively, the immunogenicity of pathogens, tumors, and self Ags, thus contributing to the education and maintenance of T cells.

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