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Multiple Knockout of Classical HLA Class II β-Chains by CRISPR/Cas9 Genome Editing Driven by a Single Guide RNA

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Comprehensive knockout of HLA class II (HLA-II) β-chain genes is complicated by their high polymorphism. In this study, we developed CRISPR/Cas9 genome editing to simultaneously target HLA-DRB, -DQB1, and -DPB1 through a single guide RNA recognizing a conserved region in exon 2. Abrogation of HLA-II surface expression was achieved in five different HLA-typed, human EBV-transformed B lymphoblastoid cell lines (BLCLs). Next-generation sequencing–based detection confirmed specific genomic insertion/deletion mutations with 99.5% penetrance in sorted cells for all three loci. No alterations were observed in HLA-I genes, the HLA-II peptide editor HLA-DMB, or its antagonist HLA-DOB, showing high on-target specificity. Transfection of full-length HLA-DPB1 mRNA into knockout BLCLs fully restored HLA-DP surface expression and recognition by alloreactive human CD4 T cells. The possibility to generate single HLA-II–expressing BLCLs by one-shot genome editing opens unprecedented opportunities for mechanistically dissecting the interaction of individual HLA variants with the immune system. ***The Journal of Immunology*****, 2019, 202: 000–000.**

CD4$^+$ T cells play a central role in adaptive immunity, both as Th cells orchestrating the humoral and cellular immune response, and as regulatory T cells mediating immunological tolerance (1). The function of these cells is critically dependent on interaction of their TCR with antigenic peptides presented by HLA class II (HLA-II) Ags on the target cell surface (2). However, the molecular analysis of this interaction is complicated by the fact that the β-chain genes of classical HLA-II molecules are encoded by three highly polymorphic loci HLA-DRB, -DQB1, and -DPB1, with a total of 4565 allelic variants reported to date (ImMunoGeneTics HLA databank, release 3.31.0) (3). A single cell can express up to eight different HLA-II specificities on the cell surface, each composed of a nonpolymorphic α-chain dimerized with a different polymorphic β-chain encoded by up to two different alleles at the loci HLA-DRB1, -DRB3/4/5, -DQB1, and -DPB1. Each of these HLA-II variants will present a different mixture of antigenic peptides to the TCR of CD4$^+$ T cells. In this scenario, artificially generated cells expressing a single HLA-II Ag at a time are invaluable tools to mechanistically dissect the interaction of individual HLA variants with CD4$^+$ T cells in different contexts, including autoimmunity and transplantation. Moreover, single HLA-II–expressing cells are crucial for the analysis of the immunopeptidome presented by individual HLA-II Ags, a subject of increasing interest, which has been lagging behind similar analyses for HLA-I, mainly because of the scarcity of appropriate strategies to generate these cells.

First attempts to this end were made in the past by using HLA-II–negative fibroblast or tumor cell lines, in which single Ag expression was achieved by transfection of HLA-II α- and β-chain genes together with other molecules necessary to provide the machinery needed for proper HLA-II processing and recognition by CD4$^+$ T cells (4–8). The major drawback of this approach is the use of nonhematopoietic cell lines, which introduces important potential variables regarding the peptidome displayed by the transfected HLA-II Ags. Moreover, this approach is not amenable to the personalized generation of single HLA-II–expressing cells from any individual.

Recent advances in the field of genome editing have allowed the development of new methods to knockout HLA-I expression by means of Zinc-finger nucleases or clustered, regularly interspaced, short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) technologies (9–12). These techniques are very promising for their potential application in cell product manipulation for cellular therapy and offer the unprecedented opportunity to develop a general method applicable to any individual for the generation of single HLA-expressing cells through the knockout of endogenous HLA and subsequent replacement with individual

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Abbreviations used in this article: BLCL, B lymphoblastoid cell line; Cas9, CRISPR-associated protein 9 nuclease; CRISPR, clustered, regularly interspaced, short palindromic repeat; ER, endoplasmic reticulum; gRNA, guide RNA; HLA-II, HLA class II; Ii, invariant chain; NGS, next-generation sequencing; WT, wild-type.

alleles (10). Because of the greater complexity of HLA-II structure and expression regulation, generation of single HLA-II–expressing cells has lagged behind HLA-I (10–12) but has recently been proposed by knockout of CIITA (9). Because CIITA regulates transcriptional expression of all classical and nonclassical HLA-II genes (13), restoration of single HLA-II expression by this approach requires transfection not only with the relevant α- and β-chain genes, but also with the nonclassical peptide chaperone HLA-DM and the invariant chain ([Ii] CD74). This is a major drawback associated with significant technical challenges and experimental variability.

In this study, we have overcome these hurdles by applying CRISPR/Cas9 genome editing to the simultaneous targeting of classical HLA-II DRB, DQB1, and DPB1, but not the nonclassical HLA-DM and DO genes with a single guide RNA (gRNA). Using EBV-transformed B lymphoblastoid cell lines (BLCLs) as model, we show that this approach allows the simple functional transfection of a single HLA-II β-chain gene.

Materials and Methods

Cells and cell lines

PBMC were obtained from healthy blood donors referred to the University Hospital Essen, after informed consent, and typed for HLA-DPB1 by medium resolution Luminex sequence-specific oligonucleotide (One Lambda, Los Angeles, CA) under standard procedures accredited by the European Federation for Immunogenetics. HLA-typed EBV-transformed BLCLs were purchased from the European Collection of Authenticated Cell Culture or locally established from PBMC. A list of BLCLs and their respective HLA types used is presented in Supplemental Table I. Hela cells expressing the Ii, HLA-DM, CD80, and single HLA-DPB1*02:01,*03:01, *04:01, or *04:02 (Hela-DP) were obtained as described (6, 7).

Flow cytometry and fluorescent mAb

Flow cytometric analysis was performed on a Gallios (Beckman Coulter) using the Kaluza software. The following mAbs were used: anti-pan–HLA-I PE (clone W6/32; Thermo Fisher Scientific, Waltham, MA), anti-pan–HLA-II PE/Cy7 (clone Tü39; BioLegend, Koblenz, Germany), anti–HLA-DR PE (clone L243; BD Biosciences, Heidelberg), anti–HLA-DQ PE (clone HLADQ1; BioLegend, Koblenz), anti–HLA-DP PE (clone B7/21; Leinco Technologies, St. Louis, MO), anti-CD4 PE/Cyanine 7 (clone SK3; BD Biosciences), anti-CD8 Pacific Blue (clone B9.11; Beckman Coulter, Krefeld), anti-CD74 PE (clone LN2; BioLegend), and anti-CD137 allophycocyanin (clone 4B4-1; BD Biosciences). The locus-specific mAbs for HLA-II were selected to be pan-specific, binding to conserved epitopes in HLA-DR (L243) (14) and HLA-DP (B7/21) (15). The HLA-DQ–specific mAb DQ1 covers all HLA-DQ specificities except for DQ2. The anti-pan–HLA-II Ab Tü39 is directed against a monomorphic epitope shared by all HLA-II Ags (16).

Lentiviral vectors

The CRISPR/Cas9 vector pCRG-g61 was designed based on the pRRLSIN backbone (17). The U6-promoter/gRNA expression unit and the EFS-promoter/Cas9 cassette were obtained from pLentiCrispr-v2 vector (18), and the puromycin resistance encoding sequence was exchanged with a GFP-coding sequence. The specific section of the gRNA-61 sequence was cloned into the Esp3I sites using complementary oligonucleotides. The alignment of the gRNA-61 target sequence in different HLA-II alleles was performed using the T-coffee algorithm (19). For stable rescue of HLA-DP expression, the coding sequence of HLA-DPB1*03:01 and *04:01 was modified by PCR-based introduction of four silent mutations into the target site of gRNA-61. The CRISPR/Cas9 target site, including the protospacer adjacent motif (PAM) sequence, was mutated from 5′-AACAGCCAGAAG-3′ to 5′-AATTCCCAGAAA-3′. The coding sequence of the two thus mutated HLA-DPB1 alleles was subsequently cloned into a bidirectional promoter lentiviral vector encoding HLA-DPB1 together with the reporter gene ΔLNGFR as previously described (20, 21). Lentiviral particles were produced in 293T cells by cotransfection of pMD2G, pCMV-dR8.9, and the lentiviral vector.

Generation of HLA-II knockout BLCLs

BLCLs were transduced with viral particles generated with the pCRG-g61 vector. Four to six days later, the cells were stained with a mixture of anti–HLA-DR (clone L243), anti–HLA-DQ (clone HLADQ1), and anti–HLA-DP (clone B7/21) PE-conjugated Abs and sorted on an FACSAria III flow cytometer (BD Biosciences), gating on cells with a strongly reduced PE fluorescence. This procedure was repeated 1 wk later.

Assessment of cell surface expression of HLA Ags in BLCLs

Cell surface staining was performed on wild-type (WT) BLCLs or after CRISPR-Cas9–mediated knockout using the above mentioned panel of HLA-specific mAbs. Median fluorescence intensity for each of the mAb was calculated on cells gated as follows: selection of singlet events on forward scatter area/height dot plots, followed by gating on forward/side scatter dot plots.

Immunoblot analysis and Abs

Immunoblot analyses were performed on total cell lysate from 5–10 × 10^6 BLCL in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) IGEPAL buffer (Sigma-Aldrich, Munich, Germany), 1% (w/v) Na-deoxycholate, 50 mM NaF, 0.1 mM Na-vanadate, 0.1% (w/v) SDS, 0.2 mM PMSF, 1 mM DTT, 1 μg/ml leupeptin and pepstatin, and cOmplete Protease Inhibitor (Roche, Basel, Switzerland). Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with the following primary Abs: anti–HLA-DP β-chain (DPβ) (polyclonal serum; Thermo Fisher Scientific), anti–HLA-DP α-chain (DPα) (mAb NB-A3), anti–HLA-DR β-chain (DRβ) (mAb DA2), anti–HLA-DR α-chain (DRα) (mAb G7), anti–HLA-DM β-chain (DMβ) (mAb E8), anti–HLA-DO β-chain (DOβ) (mAb DOB.L1), anti-CD74 (mAb LN-2), and anti-GAPDH (mAb 0411), all from Santa Cruz Biotechnology, Santa Cruz. After incubation with peroxidase-coupled secondary Ab (Jackson ImmunoResearch Laboratories, Ely, U.K.), signal detection was performed using a ECL chemiluminescence system (Cell Signaling Technology, Danvers, MA).

XBP1 quantitative RT-PCR

RNA was prepared using TRIzol (Thermo Fisher Scientific) from 1 × 10^6 MGAR WT or knockout BLCLs, and, as a positive control, WT cells were treated for 6 h with 0.5 μM thapsigargin (Sigma-Aldrich). Reverse transcription was performed with 0.5 μg total RNA (SuperScript IV; Thermo Fisher Scientific). Quantitative PCRs were performed using SYBR Green–based assays with AmpliTaq Gold (Thermo Fisher Scientific) for XBP1 (forward primer: 5′-AGCCCCTAGTCTTAGAGATACCC-3′, reverse primer: 5′-ACAGGTTCTTCCTTCACTGAGAC-3′) and XBP1s (forward primer: 5′-GGCCTTGTAGTTGAGAACCAGGA-3′, reverse primer: 5′-CCTGCACCTGCTGCGGACT-3′). All reactions were performed in triplicate. Quantitative data were calculated as the normalized ratio of relative transcript amounts based on the average of three cycle threshold values.

Next-generation sequencing–based molecular characterization of CRISPR/Cas9 mutagenesis

HLA-A, -B, and -C, -DRB, -DQB1, and -DPB1 typing was performed by next-generation sequencing (NGS) of exon 2 on an Illumina MiSeq platform according to previously described protocols (22). HLA-DMB and -DOB NGS were performed according to analogous protocols using the primers 5′-CCTTCCCTGGCTCCTCTAG-3′ and 5′-GCACCCCTCTCTCCTCAC-3′ (HLA-DMB) and 5′-GAGGGGGCTGGTTCATGG-3′ and 5′-CCTCAGCTTCCAGCTCAC-3′ (HLA-DOB), targeting the intron sequence directly upstream and downstream of exon 2 in each gene. Read depth was at least 2500 reads per HLA locus. The background of artificial reads detected as CRISPR/Cas9 modified was <0.5% in all cases obtained from negative control WT BLCLs.

For the analysis of genomic alterations generated by CRISPR/Cas9, NGS data were submitted to the online tool Cas-Analyzer (www.rgenome.net/cas-analyzer/) (23). Pairwise alignment of clustered reads to the reference WT sequence was performed to identify position and length of insertion/deletion mutations as well as reading frame changes in the coding sequence. Figures were generated by the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA).

Restoring single HLA-DP expression in BLCLs

Rescue of HLA-DP expression in knockout BLCLs was performed both transiently and stably. For transient rescue, the full-length HLA-DPB1 coding sequence was generated by PCR from cDNA of different HLA-typed BLCLs, as described (24), cloned into the vector pCR2.1 and sequenced on both strands (Microsynth Seqlab, Göttingen). HLA-DPB1 mRNA was in vitro transcribed from plasmid DNA by use of the mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific). A total

of 0.5 μg mRNA was used for transient transfection by electroporation into BLCLs using the Neon Transfection System (Thermo Fisher Scientific) according to the manufacturer's recommendations. Electroporated cells were kept in culture in standard medium for 24 h before assessment of HLA-DP expression by flow cytometry. For stable rescue, knockout BLCLs were transduced with lentiviral vectors carrying silent mutations within the target site of gRNA-61 (described in *Lentiviral vectors*). One week after transduction, rescued cells were flow sorted based on positivity for HLA-DP/ΔLNGFR cell surface expression.

$CD4^+$ T cell allorecognition of single HLA-DP–expressing BLCLs

Alloreactive T cell lines were established by stimulation of $CD4^+$ T cells from healthy donors with irradiated HeLa-DP cells in 14 d culture as previously described (6, 7). Specificity of T cells was tested by allorecognition of the original HeLa-DP stimulator cells compared with HeLa-DP carrying an autologous HLA-DPB1 allele in CD137 upregulation assays as described (6, 25). Allorecognition of BLCLs before or after CRISPR/Cas9-mediated HLA-II knockout, or after restoring HLA-DP expression by transfection, was also tested in CD137 upregulation assays. Gating strategy was the following: singlet events were gated on forward scatter area/height dot plots, followed by gating of cells on forward/side scatter dot plots; finally, $CD4^+$ T cells were gated on CD4/CD8 dot plot and analyzed for CD137 expression on CD4/CD137 dot plots.

Results

Experimental platform and gRNA-61 for multiple HLA-II β-gene knockout

We set out to generate a platform of BLCLs lacking the β-chains of all three classical HLA-DR, DQ, and DP Ags, but not of the nonclassical, HLA-DM peptide–processing chaperone and its antagonist HLA-DO to enable rescue of individual HLA-II Ag expression by simple transfection with the relevant β-chain gene (Fig. 1A). To this end, we designed a single gRNA, designated gRNA-61, recognizing a conserved target sequence encompassing nt 162–180 in exon 2 of HLA-DRB1, -DRB3, -DRB4, -DRB5, and -DQB1 and 156–174 in exon 2 of HLA-DPB1 for CRISPR/Cas9 genome editing (Fig. 1B). This region encodes aa 59–65 (57–63 in HLA-DPB1), which are located in the α helix at one side of the peptide binding groove in proximity of peptide pockets 9 and 7, and alterations in this region are therefore likely to abrogate cell surface expression even if in frame. In almost all HLA-DRB, -DQB1, and -DPB1 alleles known to date (26), the target region is complementary to at least 18 of the 19 target-specific nucleotides in gRNA-61, with 3731/4565 (81.7%) and 809/4565 (17.7%) alleles carrying no or a single mismatch, respectively (Supplemental Table II) (26). In contrast, all HLA-DMB and -DOB alleles reported so far carry at least 4 nt differences in the target region (Fig. 1B) (ImMunoGeneTics HLA databank release 3.34.0) (27), and no complementary region is found in the genes encoding Ii, HLA-DRA, -DQA1, and -DPA1 (data not shown).

Knockout of HLA-II expression in BLCLs

HLA-II knockout was tested on four HLA-II homozygous and one HLA-II heterozygous BLCLs with different HLA types (Supplemental Table I). After transduction, cells with a HLA-DR–negative phenotype were present in substantial frequencies, ranging from 1.5 to 9.7%, and could be flow sorted to >99% purity. The sorted cells were kept in culture for at least 15 d before flow cytometric analysis of HLA-II and showed an at least two-log decrease in HLA-DR, DQ, and DP cell surface expression compared with WT, whereas HLA-I expression was essentially unchanged (Fig. 2A for two representative BLCLs, Supplemental Table I for all five). Of note, one BLCL expressed three DRB alleles because of heterozygosity for DRB1*01:01 and *16:01, the latter in linkage with the additional DRB5*02:02. Complete knockout of HLA-DR cell surface expression was observed also in this BLCL (Fig. 2A). The stability of the knockout phenotype was confirmed again after more than 2 mo of culture using locus-specific (data not shown) and pan–HLA-II Abs (Supplemental Fig. 1). No difference in the knockout efficiency, as determined by cell surface expression, was observed for the alleles HLA-DRB1*07:01 and -DPB1*03:01 compared with the others despite their reduced complementarity of only 18 instead of 19 nt with the gRNA-61 target sequence (Figs. 1, 2). This observation is in line with previous reports indicating that CRISPR/Cas9 genome editing can tolerate limited numbers of mismatches in the target sequence (28–30).

The effect of CRISPR/Cas9-mediated knockout on expression of classical and related HLA-II molecules was further investigated by immunoblot analysis in knockout BLCLs compared with WT (Fig. 2B). As expected, HLA-DR and -DP β-chain expression was detected in WT, but abrogated in knockout cells, whereas expression of HLA-DM and -DO β-chain was unaffected. The amount of both HLA-DR and -DP α-chain was markedly reduced in knockout compared with WT BLCL despite similar transcript levels for HLA-DPA1 and -DPB1 (data not shown), suggesting instability of free α-chain in the absence of the β-chain. In contrast, there was a slight increase of CD74 (Ii) in knockout compared with WT BLCL, which, however, did not translate into significant changes in the CD74 cell surface expression (Fig. 2C). Finally, to test if HLA-II β-chain knockout induces endoplasmic reticulum (ER) stress, we tested for inositol-requiring enzyme 1 (IRE1) activity. This protein unconventionally splices the transcription factor XBP1 mRNA in the cytoplasm to an XBP1 isoform (XBP1s) involved in the ER stress response (31, 32). Although thapsigargin-treated positive control BLCL showed a significant induction of XBP1s as an indicator for ER stress, no evidence of such an increase was observed in knockout compared with WT BLCL (Fig. 2D).

Mapping of HLA-II CRISPR/Cas9 genome editing by NGS

CRISPR/Cas9 genome editing generates DNA double-strand breaks at the target site, inducing the activation of nonhomologous end-joining pathway to repair the damage (33). This error-prone process leads to the introduction of insertion/deletion mutations of variable lengths into the coding reading frame of the gene of interest, with variable degrees of off-target activity, depending on the specificity of the gRNA (33). To investigate target specificity of our CRISPR/Cas9 HLA-II β-chain gene knockout, we performed NGS of exon 2 for HLA-DRB, -DQB1, -DPB1, -DMB, and -DOB, and HLA-I A, B, and C as negative control, on five knockout BLCLs after flow sorting (Supplemental Table I). The frequency distributions were similar for all five BLCLs, with one representatively shown in Fig. 3. The mean read depth among all HLA loci for this BLCL was 5809 ± 2704, ranging from 2545 reads for HLA-DPB1 to 9628 reads for HLA-C. As expected, the frequency of alterations in the HLA-I genes was extremely low and showed a random distribution pattern, (<0.05% of the total reads) throughout exon 2, which is comparable to the error rate of PCR amplifications (22). In HLA-DRB, -DQB1, and -DPB1, almost all detected sequences (>99.0% of total reads) contained either insertions (18.19 ± 6.01%) or deletions (81.35 ± 5.94%) around the target break point (position 175 for HLA-DRB and -DQB1 genes or 169 for HLA-DPB1). In line with previous reports (28), the majority of detected alterations consisted in deletions of variable length ranging from 1 to 158 bp, with the most frequent deletion regarding a single bp at position 176 (170 of HLA-DPB1) present in 20.9 ± 4.8% of deletions. Insertions were less variable and mainly consisted in the introduction of a single

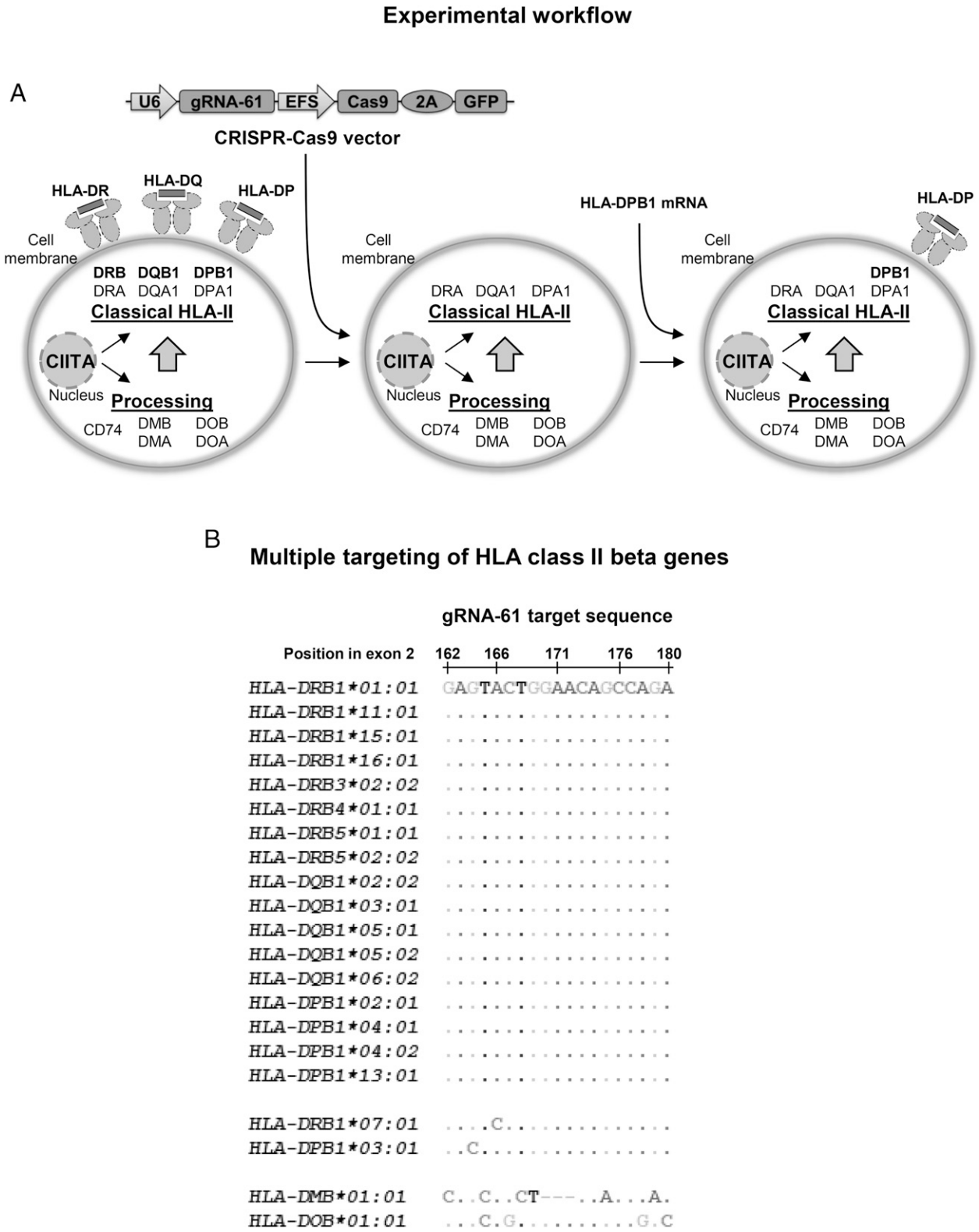

FIGURE 1. Generation of single HLA-DP–expressing BLCLs by multiple CRISPR/Cas9 knockout of HLA-II β-chain genes. (**A**) Experimental workflow. WT BLCLs are transduced with a Cas9 vector coexpressing the gRNA-61 and GFP and flow sorted for the GFP$^+$/HLA-II phenotype to generate knockout BLCLs. Cells expressing a single HLA-DP Ag are obtained by transfection of knockout cells with in vitro synthetized HLA-DPB1 mRNA, followed by flow-sorting of GFP$^+$/HLA-DP$^+$ cells. (**B**) DNA target sequence of gRNA-61 in HLA-II β-chain genes. Sequence alignment is shown for the 19 HLA-DRB, -DQB1, -DPB1, and two HLA-DMB and -DOB alleles carried by the five BLCLs used for CRISPR/Cas9-mediated HLA-II knockout in this study (see Supplemental Table I), with HLA-DRB1*01:01 as reference. Note that 17/19 classical β-chain genes listed are perfectly complementary to the gRNA-61 target sequence, and two of them carry a single mismatch, whereas the two nonclassical β-chain genes carry four or more mismatches. Sequences were aligned using the T-coffee algorithm (19); points and dashes represent sequence identity and missing nucleotides, respectively. The different nucleotides are indicated in different shades of grey. The gRNA-61 target sequence corresponds to position 162-180 in exon 2 of HLA-DRB, -DQB1, and -DOB, to position 156 to 174 in HLA-DPB1 and to position 177 to 192 in HLA-DMB.

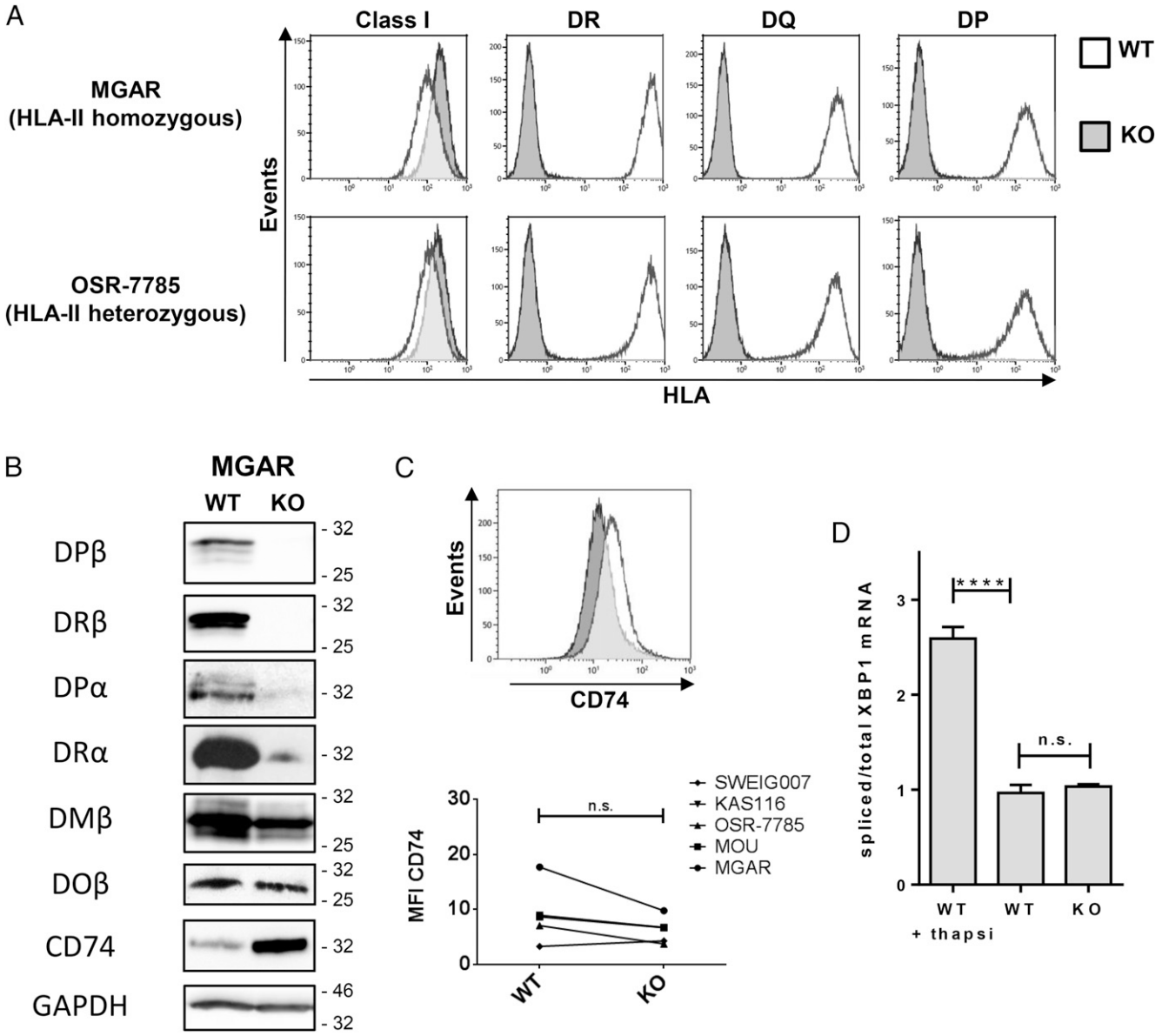

FIGURE 2. Characterization of BLCLs after CRISPR/Cas9-mediated knockout of HLA-II β-chain genes. (**A**) Cell surface staining is shown for HLA-DR (mAb L243), -DQ (mAb HLA-DQ1), and -DP (mAb B7/21) or HLA-I (mAb W6/32), for two representative BLCLs, MGAR and OSR-7785, before (WT) or after CRISPR/Cas9 knockout (KO) of the HLA-II β-chain genes as described in *Materials and Methods*. Genome-edited BLCLs were flow sorted for the HLA-DR–negative phenotype and expanded for at least 15 d before flow cytometric analysis. Note that OSR-7785 carries the allele HLA-DPB1*03:01 (see Supplemental Table I), which is complementary to only 18 of 19 nt in the gRNA-61 target sequence, yet shows a complete knockout of HLA-DP after genome editing. (**B**) Immunoblot analysis of classical and accessory HLA-II molecules in WT and KO BLCLs. Total cell lysates were separated by 10% SDS-PAGE and probed for the indicated molecules as described in Material and Methods. GAPDH was detected after stripping and reprobing each membrane with anti-GAPDH Ab as control; a representative result is shown in the bottom panel. Sizes in kilodaltons are reported on the right side of each panel. Similar results were obtained for other two tested BLCLs. (**C**) Cell surface expression of CD74 was measured on the cell surface of WT and KO BLCLs by flow cytometric staining with the mAb LN2. Shown is a representative histogram plot for BLCL MGAR WT (white) or KO (gray) (upper panel), and the mean median fluorescence intensity (MFI) of three independent measurements was performed on the five different BLCL (lower panel). No significant differences in the mean MFI was observed by the paired *t* test (mean ± SD, 9.1 ± 5.3 in WT versus 6.2 ± 2.4 in KO). $p = 0.1169$. Mean ± SD MFI of the isotype controls were 0.57 ± 0.13 and 0.58 ± 0.08 for WT and KO, respectively. (**D**) A total of 1×10^6 MGAR cells were cultured for 6 h with or without thapsigargin (thapsi; 0.5 μM), and relative amounts of XBP1 and the IRE1-spliced form of XBP1 (XBP1s) were determined by quantitative RT-PCR. Normalized ratio of XBP1s to total XBP1 mRNA is shown as mean ± SD of three independent experiments. Statistical comparison was performed by the unpaired *t* test. $****p < 0.0001$.

nucleotide at position 176 (170 of HLA-DPB1) in 82.7 ± 7.0% of insertions. Together, the number of alterations leading to a change of the reading frame was 85.5 ± 5.1% of the total.

In contrast to the classical HLA-II β-chain genes, the nonclassical HLA-DM and DO showed similar patterns to HLA-I (i.e., almost all the detected reads contained WT sequences with only a very low percentage of alterations scattered throughout exon 2; Fig. 3). These data show that, as predicted by sequence alignment of the gRNA-61 target, our CRISPR/Cas9 β-chain gene knockout did not affect the nonclassical chaperone genes HLA-DM and -DO. Both are important modulators of HLA-II peptide processing, HLA-DM by restricting the peptide binding repertoire to high affinity peptides, and HLA-DO by acting as antagonist of HLA-DM in certain cell types, including BLCLs (34). Our data

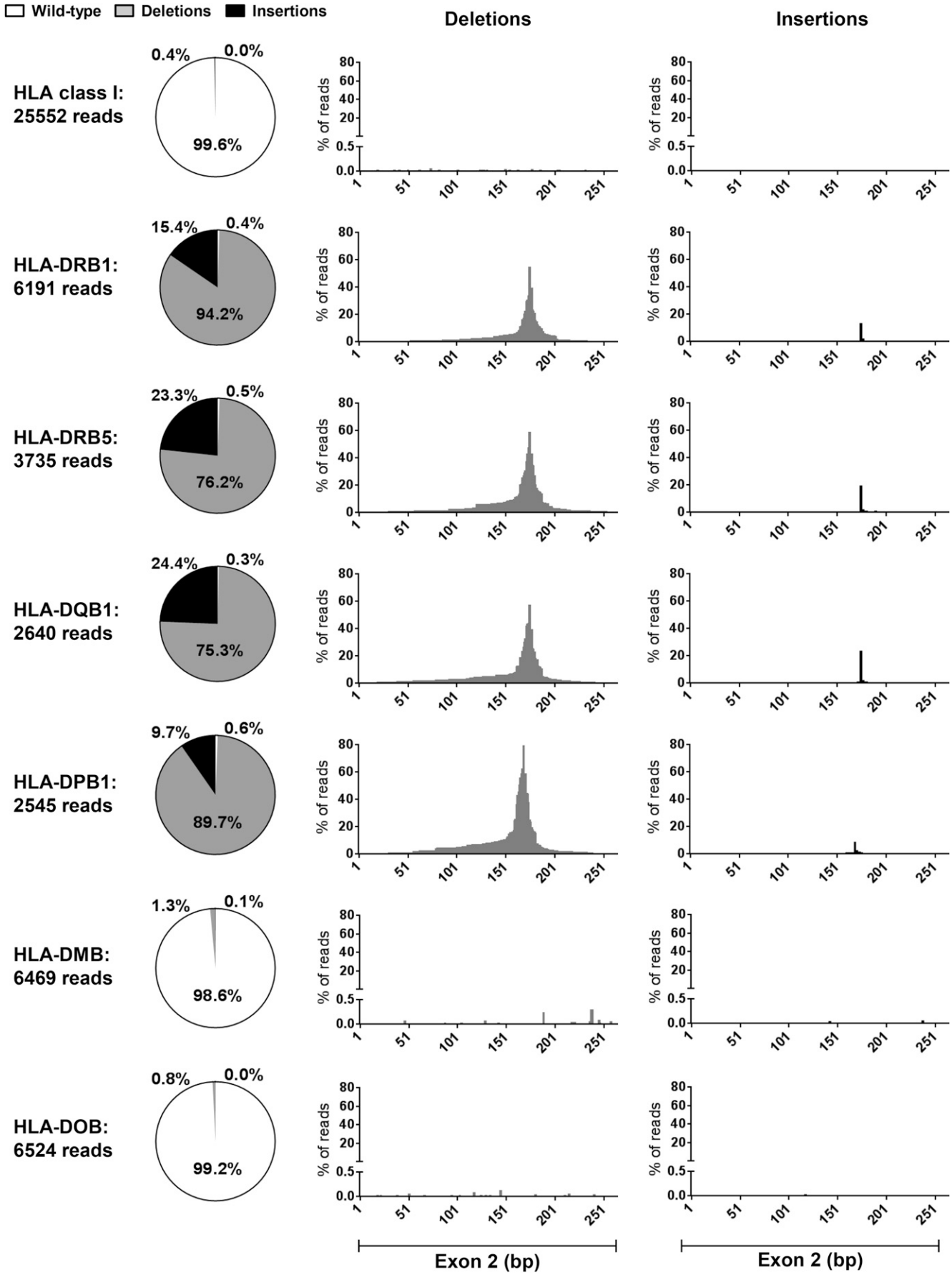

FIGURE 3. Mapping HLA-II CRISPR/Cas9 genome editing by NGS. Genomic DNA was extracted from flow-sorted and expanded HLA-II knockout MGAR cells (Fig. 2) and subjected to targeted NGS of exon 2 of HLA-A, -B, and -C, -DRB1, -DQB1, -DPB1, -DMB, and -DOB, as described in *Materials and Methods*. Reads encompassing the gRNA-61 target sequence were aligned with WT and analyzed using the online webtool *(Figure legend continues)*

show HLA-DM and -DO need not be reintroduced to restore physiological HLA-II processing after knockout of the classical β-chain genes by our approach.

Rescue of HLA-DP expression in HLA-II knockout BLCLs restores T cell allorecognition

To investigate whether single HLA-II expression can be functionally rescued in BLCLs after CRISPR/Cas9 targeting of HLA-II β-chain genes, knockout BLCLs were transiently transfected with full-length HLA-DPB1 mRNA. This restored high levels of HLA- DP cell surface expression in over 80% of cells, in four different BLCLs, and with different HLA-DPB1 alleles tested (Fig. 4A, 4C). Each BLCL was transfected with two HLA-DPB1 alleles, one corresponding to the endogenous WT allele of the BLCL, and the other one being autologous to the $CD4^+$ T cells used to obtain alloreactive responses against that WT allele. The alloreactive $CD4^+$ T cells were raised by coculture with irradiated HLA-DPB1*04:01–, *04:02–, *02:01–, and *03:01–expressing cells, and their specificity was demonstrated in CD137 upregulation assays (at least 27% of specific alloresponse; data not shown). All tested T cell effectors were able to efficiently recognize their alloantigen endogenously expressed by the WT BLCL, but completely failed to recognize the HLA-II knockout. Restauration of HLA-DP cell surface expression by the knockout BLCL resulted in complete and specific rescue of T cell alloreactivity, as demonstrated by recognition of knockout BLCL after transfection with an mRNA encoding the allogeneic, but not the autologous, HLA-DPB1 allele (Fig. 4B, 4D). Similar data were also obtained after stable lentiviral vector–mediated rescue of HLA-DP expression in BLCLs (Supplemental Fig. 2). In this case, gRNA targeting was abrogated by the introduction of four silent nucleotide mutations into the HLA-DPB1 coding region as described in *Materials and Methods*. Taken together, these data demonstrate reversibility of the HLA-II knockout by simple transfection with a selected HLA-II β-chain gene, resulting in the generation of functional single HLA-DP–expressing BLCLs.

Discussion

We have developed an innovative approach for the generation of BLCLs expressing single HLA-II cell surface molecules in the context of an intact processing machinery from virtually any individual. Although BLCLs are not ideal APCs for in vitro priming of immune responses because of the strong EBV-specific responses they elicit, they remain very valuable tools for studying MHC restriction of Ag-specific T cells, an issue of increasing importance also in vaccination trials for viral or tumor Ags (35–39). For this purpose, one has to rely generally on the availability of third-party BLCLs homozygous for individual MHC alleles. With the method presented in this study, single HLA-II–presenting BLCLs can be generated at will from any individual by simple transfection of the β-chain of interest. This will also be useful for alloantigen-specific T cells, because minor histocompatibility Ag targets of T cell alloreactivity have unforeseeable interindividual variation and may not be present on third-party BLCLs, even if they share the relevant MHC class II restriction element (40). Our method will enable the investigation of allorestricted T cell responses in the same genetic background used for priming the response. Another useful application of our method to BLCLs regards their being a rich source of HLA molecules complexed with peptides that can be used for studying the immunopeptidome presented by individual HLA-II specificities. In the rapidly evolving field of HLA immunopeptidomics, analysis of HLA-II has lagged behind HLA-I partly because of the scarcity of appropriate cells expressing single HLA-II variants (41–43). Our method provides an elegant solution to this problem by simple transfection of a knockout BLCLs with a single β-chain of interest. This approach will allow researchers to study the immunopeptidome of virtually any HLA-II Ag of interest and of in vitro generated mutants thereof. Moreover, also in this context, the possibility to generate personalized, single HLA-II–expressing BLCLs of any individual will make it possible to address questions concerning the modulation of the immunopeptidome in different genetic backgrounds. This is likely to foster research in this field of increasing relevance for immunology, including tumor immunology, autoimmunity, and infectious diseases.

A particular asset of our strategy relies in the fact that it simultaneously targets multiple endogenous HLA-II Ags within and across the three loci HLA-DR, DQ, and DP, thereby eliminating the problems associated with heterozygosity and linkage disequilibrium in HLA-II. The latter is especially relevant for HLA-DR, because many DRB1 alleles are in tight linkage disequilibrium with DRB3, DRB4, or DRB5, giving rise to up to four different HLA-DR Ags coexpressed by the same cell. The functional dissection of the relative role of individual HLA-DR Ags is severely hampered by this genetic particularity (43, 44) and can be overcome in a straightforward and efficient way by the one-shot CRISPR/Cas9 genome editing we developed.

The complete knockout of HLA-I has been reported using six different gRNAs targeting exon 2 and 3 of HLA-A, B, and C (10). In our design, the use of a single gRNA targeting all HLA-II β-chain genes at once greatly reduces the chance of potential off-target effects of CRISPR/Cas9 genome editing, as confirmed by the observation that nonclassical HLA-II differing for only 4 nt in the gRNA target sequence were not affected. Moreover, the use of a single vector has significant technical advantages by reducing handling time and complexity and could potentially also help to overcome regulatory hurdles for clinical applications in cellular therapy. A current limitation of our protocol is the relatively low initial knockout efficiency, which requires sorting and subsequent expansion of cells with the desired phenotype, making it difficult to apply to primary cells with limited proliferative potential, such as dendritic cells or hematopoietic stem cells. This difficulty might be less prominent if Cas9 ribonucleoprotein was to be delivered by transient transfection rather than, as in our approach, by stable lentiviral vector–mediated transfer, which is associated with cell type–dependent impediments to efficiency. Improvements in the vector delivery are warranted to overcome these hurdles.

In conclusion, the method we developed will prove useful for the study of HLA-II Ags and their interaction with the immune system in many different settings. These include, but are not limited to, the in vitro functional analysis of $CD4^+$ effector and regulatory T cells restricted to any single HLA-II molecule of choice, an important tool for addressing numerous questions in autoimmunity, cancer immunology, and transplantation. Moreover, single HLA-II–expressing

Cas-analyzer (23). Pie charts show the percentage of reads complementary to the exon 2 WT sequence (white) or containing insertions (black) or deletions (gray). Histograms show the position of insertion and deletion mutations within the 270 bp of the exon 2. The gRNA-61 target sequence encompasses position 162 to 180 in exon 2 of HLA-DRB1 and -DQB1 and position156 to 174 in exon 2 of HLA-DPB1. Note that virtually all insertions and deletions are within or around that sequence.

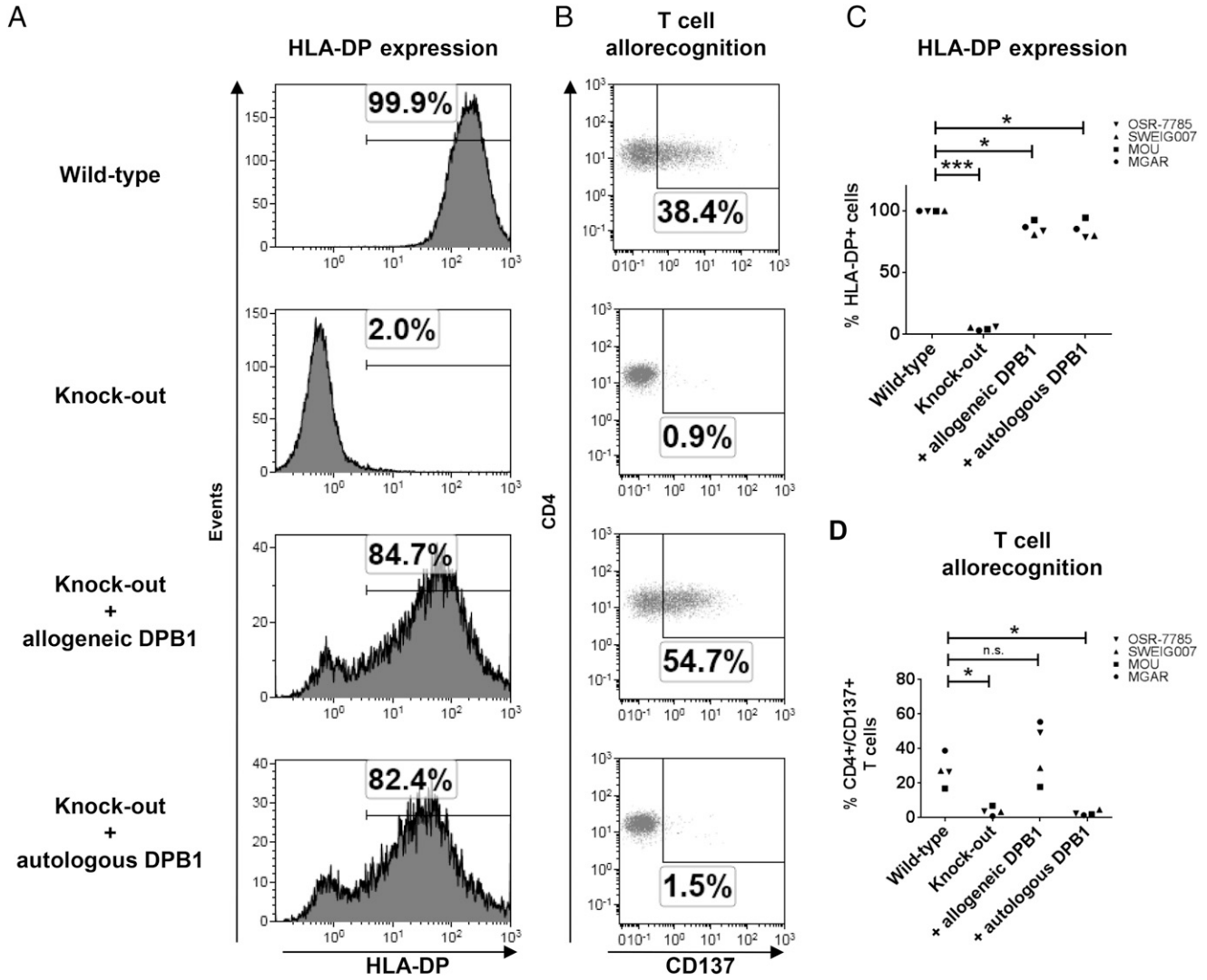

FIGURE 4. Rescue of HLA-DP expression and recognition by alloreactive T cells in HLA-II knockout BLCLs. Rescue was performed by transient transfection with full-length HLA-DPB1 mRNA, as described in *Materials and Methods*, and cells were tested after 24 h (peak of expression, which was stable up to 48 h and declined to 50% after 96 h in kinetic experiments; data not shown). (**A**) Representative flow cytometry plots of HLA-DP cell surface expression by mAb B7/21 on MGAR BLCL before or after rescue of allogeneic (HLA-DPB1*04:01) or autologous (HLA-DPB1*03:01) to T cells from the responder shown in (**B**). (B) Allorecognition of the same MGAR BLCL as in (A) by $CD4^+$ T cells from a healthy donor after 14 d coculture with irradiated Hela-DPB1*04:01, as described in *Materials and Methods*. CD137 upregulation in responder T cells, after overnight incubation with the indicated target MGAR BLCL, was determined by flow cytometry. Note similar levels of allorecognition of WT MGAR expressing endogenous HLA-DPB1*04:01 and of knockout MGAR ectopically re-expressing HLA-DPB1*04:01. (**C**) Dot plots showing HLA-DP cell surface expression levels for four different BLCLs, as indicated. (**D**) Dot plots showing T cell allorecognition of four different BLCLs, as indicated. Allogeneic and autologous HLA-DPB1 were as follows (see also Supplemental Table I): OSR-7785 HLA-DPB1*03:01 and HLA-DPB1*04:01, respectively; SWEIG HLA-DPB1*04:02 and HLA-DPB1*03:01, respectively; MOU HLA-DPB1*02:01 and HLA-DPB1*03:01, respectively; MGAR HLA-DPB1*04:01 and HLA-DPB1*03:01, respectively. Statistical analysis were performed with repeated measures one-way ANOVA between WT as reference and knockout cells before or after rescue of HLA-DP expression. $*p < 0.05$, $***p < 0.001$.

BLCLs can also be envisaged to be useful for the study of HLA-II interaction with receptors other than the TCR, such as viral receptors in the context of HIV research. As discussed above, the possibility to expand BLCLs expressing functional single HLA-II molecules in the context of an intact processing machinery also opens new avenues to the molecular characterization of the peptidome displayed by individual HLA-II Ags, a burning question fueled by the galloping recent advances in cancer immunotherapy. Finally, in a longer term perspective, our method might also prove useful in clinical protocols of personalized cellular therapy, for instance, for the generation of HLA-II–compatible hematopoietic stem cells for transplantation (45) or for the reduction of organ graft rejection by HLA-II knockout endothelial cells as previously suggested (9, 46).

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Disclosures

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