HLA-C*04:09N is expressed at the cell surface and triggers peptide-specific T-cell activation

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Received: June 23, 2023.
Accepted: September 18, 2023.
Early view: September 28, 2023.

https://doi.org/10.3324/haematol.2023.283812

Abstract

The null allele HLA-C*04:09N differs from HLA-C*04:01 in a frameshift mutation within its cytoplasmic domain, resulting in translation of 32 additional amino acids that are assumed to prevent cell surface expression. However, we recently identified a multiple myeloma-reactive T-cell receptor (TCR) that appeared to recognize antigen presented on HLA-C*04:09N and encouraged us to ask whether HLA-C*04:09N, albeit not easily detectable at the cell surface, can present antigen sufficient for T-cell activation. We generated two HLA-class I-deficient cell lines, re-expressed HLA-C*04:09N, detected HLA expression by flow cytometry, and tested for T-cell activation using a cytomegalovirus peptide-specific HLA-C*04:01-restricted TCR. In both cell lines, HLA-C*04:09N expression was detectable at the cell surface and could be enhanced by IFN-γ exposure. Recombinant HLA-C*04:09N expression was sufficient for T-cell activation *in vitro*, which could be blocked by an HLA-class I-specific antibody, suggesting HLA-TCR interaction at the cell surface. Peripheral blood mononuclear cells isolated from an individual who physiologically expressed HLA-C*04:09N triggered peptide-specific T-cell activation, confirming our results with cells with natural HLA expression levels. In conclusion, we present peptide-specific HLA-C*04:09N-restricted T-cell activation and suggest consideration of this allele in the appropriate clinical context, such as allogeneic stem cell transplantation, or in the setting of cellular therapy.

Introduction

HLA-C*04:09N (C*04:09N) is one of 344 currently reported null alleles at the HLA-C locus (https://hla.alleles.org). Accounting for frequencies between 0.00002 and 0.0189 in cohorts that include more than 1,000 individuals,[1,2] C*04:09N is considered one of the most frequent null alleles.[3,4]

C*04:09N differs from HLA-C*04:01 (C*04:01) in a point deletion within exon seven, which results in a frameshift and extension of the reading frame 97 base pairs beyond the canonical stop codon, translating into a protein 32 amino acids longer than C*04:01.[5-7] The additional 32 amino acids do not inhibit intracellular protein expression but were suggested to prevent cell surface expression.[5,7] Consequently, C*04:09N expression has been reported to be detectable by intracellular staining but not at the cell surface using the pan HLA-class I-specific antibody, clone W6/32, which resulted in its designation as a null allele.[5] The extracellular parts of C*04:09N and C*04:01 are identical, suggesting potential presentation of identical peptide repertoires.

We recently identified a multiple myeloma-reactive T-cell receptor (TCR) that appeared to recognize antigen presented on C*04:09N,[8] calling its status as a null allele into question. Current evidence that defines C*04:09N as a null allele relies on flow cytometry-based detection of HLA-cell surface expression using one HLA-class I-specific antibody clone (W6/32) and lacks functional proof of the biological irrelevance of this allele.

Our observations in multiple myeloma encouraged us to study whether C*04:09N, albeit not detectable at the cell surface in previous studies, can present antigen in a form that enables peptide-specific T-cell activation.

We asked experimentally whether C*04:09N expression: i) is detectable at the cell surface with current technologies; ii) can be modulated by the cytokine milieu; and iii) is sufficient for peptide presentation and specific T-cell activation.

Methods

Primary cells

The study was approved by the local institutional review board (protocol EA2/096/15 to LH). All participants provided written informed consent, and all research was conducted in accordance with the Declaration of Helsinki.

Cell lines and culture conditions

HEK293T (RRID: CVCL_0063) and U2OS (RRID: CVCL_0042) were cultivated in DMEM with 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, and 10 mg/mL streptomycin (all Thermo Fisher Scientific) at 37°C and 5% CO_2. If indicated, 1,000 U/mL IFN-γ (PeproTech) were added for 24 hours (h) prior to analysis or co-culture experiments.

T-cell receptor expression on human peripheral blood lymphocytes

Recombinant TCR expression in human lymphocytes was performed as previously described.[8-10] Detailed information on the QYD peptide-specific TCR has been reported previously.[11]

HLA knockout using CRISPR-Cas9

HLA-A, HLA-B, and HLA-C knockout of U2OS was performed as previously described[8] and confirmed by flow cytometry.

Recombinant HLA expression

Coding sequences of C*04:09N and C*04:01 (accession numbers HLA01451, HLA00420) were downloaded from IPD-IMGT/HLA (https://www.ebi.ac.uk/ipd/imgt/hla/), synthesized (GeneArt, Life Technologies), and cloned into the pHSE3' vector under control of an H-2K^b promoter, as previously described.[12] Cell lines were transfected with FuGENE® HD (Promega). For stable expression, cells were selected with 3 mg/mL G418 (Cayman Chem), and single cell clones were generated by limiting dilution.

Minigene expression

The amino acid sequence of cytomegalovirus (CMV)-derived pp65 was downloaded (https://www.ncbi.nlm.nih.gov, Gene ID: 3077579) and two 38 amino acid long sequences containing QYDPVAALF (QYD, underlined: MGQQIFLEVQAIRETVELRQYDPVAALFFDIDLLLQRG) and KDVALRHVV (KDV, underlined: MTSAFVFPTKDVALRHVVCAHELVCSMENTRATKMQVIG) were reverse translated using EMBOSS.[13] Minigene constructs were synthesized (Integrated DNA Technologies Inc.), cloned into pcDNA6/V5-His A, and transfected with FuGENE® HD (Promega) 36 h before co-culture experiments.

Flow cytometry

Reagents for determination of HLA expression or T-cell activation upon co-incubation are listed in the *Online Supplementary Appendix*. For intracellular staining, we used the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences). All reagents were used according to the manufacturer's instructions.

Magnetic-activated cell separation

For enrichment of CD8$^+$ T cells before co-culture experiments, we used MojoSort™ Human CD8 Nanobeads (Biolegend) for magnetic-activated cell separation (MACS) with minor modifications: CD8 Nanobeads were diluted 1:5 and cells were separated on MS columns (Miltenyi). Enriched populations were on average 71.7% CD8$^+$, as determined by flow cytometry.

Co-cultures

Co-culture experiments of TCR-transduced T cells with HEK293T or U2OS were performed in 250 μL medium (RPMI-1640 supplemented with 10% FBS, 10,000 U/mL penicillin, and 10 mg/mL streptomycin) in 48-well plates for 18 h; co-cultures with PBMC were performed in 150 μL medium in 96-well plates for 24 h at 37°C and 5% CO_2. Indicated peptides (Genscript) were added at 5 μM final concentration 1 h before co-culture. For HLA-blocking, Ultra-LEAF Purified Anti-Human HLA-class I (clone W6/32, BioLegend, RRID: AB_2561492) or Ultra-LEAF Purified Mouse IgG2a κ isotype control (clone MOPC-173, BioLegend, RRID: AB_11148947) were added at 50 μg/mL final concentration 1 h before co-culture. Activation of T cells was detected using the Human IFN-γ ELISA Set BD OptEIA (BD Biosciences, RRID: AB_2869029), the human granzyme B DuoSet ELISA kit (R&D Systems), and by flow cytometry.

HLA typing of the sample MM160

HLA-typing was performed using Holotype HLA24/7 (Omixon; Budapest, Hungary) in combination with MiSeq (Illumina; San Diego, CA, USA) according to the manufacturer's instructions. Sequencing data were analyzed using Twin software v.3.1.1 (Omixon) and IMGT/HLA-Database v.3.32. HLA-C*04:09N was confirmed by sequence-specific primer (SSP) PCR (Olerup SSP® HLA-C*04:09N, Olerup SSP AB; Stockholm, Sweden) according to the manufacturer's instructions.

Statistical analysis

Statistics were calculated using R, v.4.0.3.[14] (See figure legends for the tests applied.)

Results

HLA-C*04:09N is expressed at the cell surface

We transfected HEK293T that lack HLA-A, HLA-B and HLA-C expression (HEK$_{HLA-KO}$)[8] with pHSE3' expression plasmids that encoded C*04:09N or C*04:01 as a positive control. HLA expression was determined by flow cytometry after intracellular or cell surface staining with antibodies specific for HLA-BC (clone B1.23.2) or pan HLA-class I (clone W6/32) (*Online Supplementary Table S1*). As expected, C*04:09N was detectable by intracellular staining (Figure 1A). How-

ever, we also detected C*04:09N cell surface expression after staining with the HLA-BC-specific antibody (Figure 1A, top). In line with previous observations,[5,7] only residual C*04:09N cell surface expression could be observed with the pan HLA-class I antibody (Figure 1A, bottom). C*04:01 expression was detectable by intracellular and cell surface staining (Figure 1A).

Recombinant HLA expression can vary in the context of different cell types and due to transfection efficiency. To confirm our findings, we generated an osteosarcoma cell line deficient in HLA-A, HLA-B and HLA-C ($U2OS_{HLA\text{-}KO}$), of which we established a single cell clone-derived cell line that stably expressed C*04:09N as the only HLA-ABC allele ($U2OS_{04:09N}$). In line with our results from transient HLA expression in $HEK_{HLA\text{-}KO}$, surface expression of C*04:09N was weak but detectable with the HLA-BC-specific, but not with the pan HLA-class I-specific antibody (Figure 1B).

Microenvironmental influences including, but not limited to the cytokine milieu, can modulate HLA expression. As an example, we determined HLA-cell surface expression of $U2OS_{04:09N}$ after exposure to IFN-γ *in vitro*. While C*04:09N cell surface expression was detectable on an average of 21.4% of $U2OS_{04:09N}$, expression increased to an average of 81.8% after IFN-γ treatment. (See Figure 1C for a representative example). On average, HLA-cell surface expression on $U2OS_{04:09N}$, determined as allophycocyanin (APC) mean

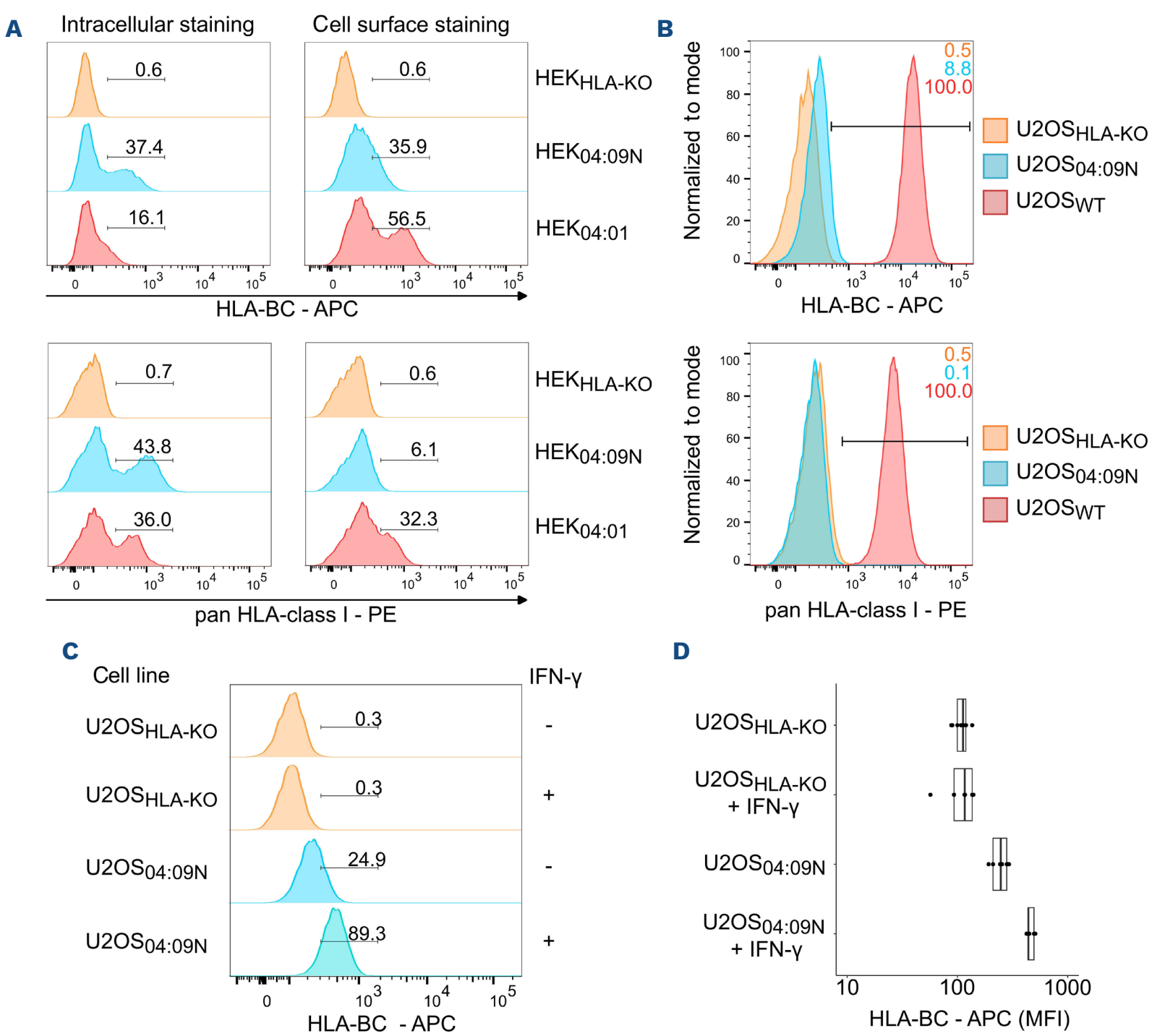


Figure 1. Detection of HLA-C*04:09N cell surface expression by flow cytometry. (A) $HEK_{HLA\text{-}KO}$ were transfected with plasmids encoding C*04:09N ($HEK_{04:09N}$) or C*04:01 ($HEK_{04:01}$) and stained with HLA-BC (clone B1.23.2, top) or pan HLA-class I (clone W6/32, bottom) antibody for flow cytometry. The plots show one representative example out of 3 independent experiments. (B) Flow cytometry detection of cell surface C*04:09N expression on $U2OS_{04:09N}$ with HLA-BC (top) or pan HLA-class I antibody (bottom). (C) Effects of *in vitro* IFN-γ exposure on C*04:09N cell surface expression on $U2OS_{04:09N}$. If indicated, cells were incubated with 1,000 U/mL IFN-γ for 24 hours (h) prior to analysis. The flow cytometry plot is representative for 3 independent experiments. (D) HLA cell surface expression of $U2OS_{04:09N}$ after 24-h IFN-γ exposure determined as mean fluorescence intensity (MFI) in 3 independent experiments with 3 replicates per experiment. Data points indicate 9 individual replicates. WT: wild type.

fluorescence intensity (MFI), increased 1.9-fold upon IFN-γ exposure (Figure 1D). IFN-γ exposure did not substantially affect cell surface staining of C*04:09N with the pan HLA-class I-specific antibody (*Online Supplementary Figure S1*). Taken together, C*04:09N expression was detectable at the cell surface of two different cell lines and increased upon IFN-γ exposure *in vitro*.

T cells can be activated by peptides presented on HLA-C*04:09N

Identical extracellular domains, including the peptide binding grooves, of C*04:09N and C*04:01 suggest binding of a similar peptide repertoire. To determine potential T-cell activation by peptides presented on C*04:09N, we expressed a C*04:01-restricted TCR specific for the CMV-derived epitope QYDPVAALF (QYD),[11] which is presented on C*04:01, in human peripheral blood T cells (T_{QYD}) (*Online Supplementary Figure S2*). (See Table 1 for HLA-class I alleles of the donor used for TCR expression). IFN-γ and granzyme B secretion were measured to detect T-cell activation. For antigen presentation, $HEK_{HLA\text{-}KO}$ were co-transfected with expression plasmids encoding C*04:09N and a minigene encoding the target epitope (QYD) or a negative control epitope (KDVALRHVV, called KDV). Only $HEK_{HLA\text{-}KO}$ co-transfected with C*04:09N and the target epitope QYD activated T_{QYD}, confirming activation of a C*04:01-restricted TCR by a peptide presented on C*04:09N (Figure 2A). Non-TCR-transduced T cells could not be activated by $HEK_{04:09N}$ confirming TCR-dependent T-cell activation (*Online Supplementary Figure S3*). T-cell activation was blocked by pan HLA-class I-specific antibody, which suggested that C*04:09N-T-cell interaction occurred at the cell surface (Figure 2B).

To confirm our results in a different cell line, $U2OS_{HLA\text{-}KO}$ and $U2OS_{04:09N}$ were transfected with the QYD or KDV minigenes and co-cultured with T_{QYD}. Only $U2OS_{04:09N}$ expressing the QYD minigene activated T_{QYD} (Figure 2C). In line with our results from HEK293T, non- transduced T cells were not activated by $U2OS_{04:09N}$ (*Online Supplementary Figure S4*). T_{QYD} activation by $U2OS_{04:09N}$ could be blocked by the pan HLA-class I antibody (Figure 2D).

Our data functionally confirmed: i) C*04:09N-dependent; and ii) target epitope-specific T-cell activation.

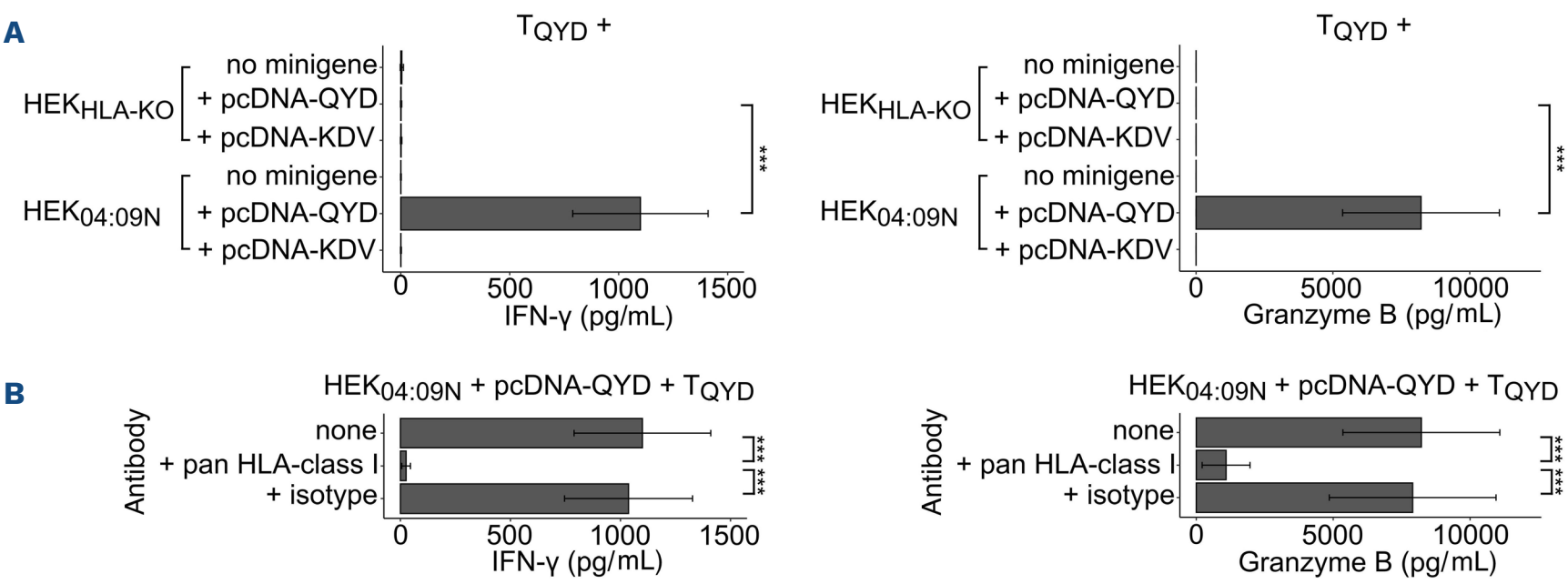
Table 1. HLA-class I alleles of the T-cell donor for T-cell receptor expression and multiple myeloma patient MM160.

Patient/Donor	HLA-class I alleles
T-cell donor	A*03:01:01; A*34:02:01 B*07:02:01; B*14:01:01 C*07:02:01; C*08:02:01
MM160	A*02:01:01G; A*23:01:01 B*44:03:01G; B*51:01:01G C*04:09N; C*15:02:01

T cells for recombinant T-cell receptor expression were isolated from leftover materials of an allogeneic stem cell graft (T-cell donor).

Peripheral blood mononuclear cells of an HLA-C*04:09N+ donor can present QYD and trigger specific T-cell activation

We obtained peripheral blood mononuclear cells (PBMC) of one multiple myeloma patient (MM160) who physiologically expressed C*04:09N (Table 1) and had been part of a previous study of our group.[8] PBMC of MM160 were rested or incubated with IFN-γ for 24 h, washed, loaded with QYD or the negative control peptide KDV, and incubated with T_{QYD}. CD137 expression on TCR-transduced $CD8^+$ T cells (see *Online Supplementary Table S2* for antibody panel and *Online Supplementary Figure S5* for gating strategy) and granzyme B in cell culture supernatants were measured to detect T-cell activation. T_{QYD} were activated by PBMC of patient MM160 in a QYD-specific manner, and activation could be enhanced by pre-incubation of PBMC with IFN-γ (Figure 3A). Accordingly, increased frequencies of dead cells among target peptide-loaded PBMC after co-culture suggested peptide-specific killing in the context of C*04:09N (Figure 3A,



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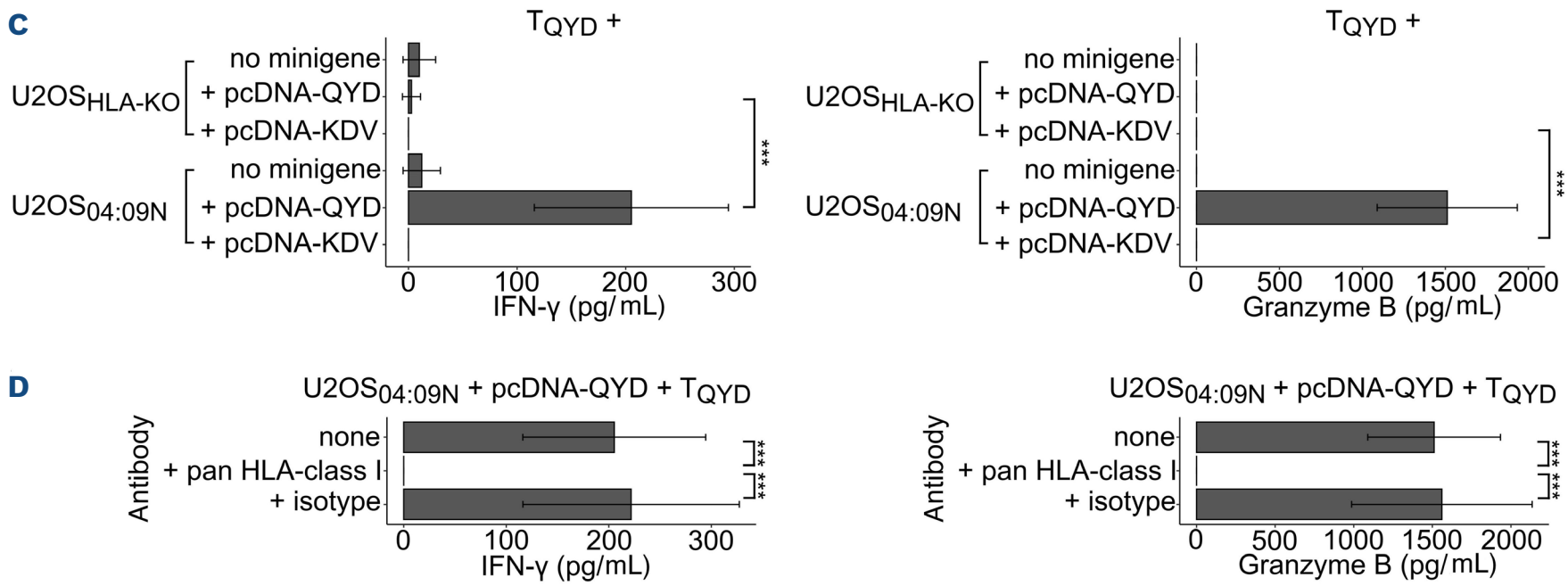

Figure 2. Specific T-cell activation by a peptide presented on HLA-C*04:09N. (A) T_{QYD} were cultured with HEK_{HLA-KO} which were (co-)transfected with a plasmid encoding C*04:09N and a minigene encoding QYD or KDV. (B) T_{QYD} were incubated with HEK_{HLA-KO} co-transfected with a plasmid encoding C*04:09N and the QYD-minigene in presence of a blocking antibody against pan HLA-class I (clone W6/32) or isotype control (clone MOPC 173). (C) T_{QYD} were incubated with $U2OS_{HLA-KO}$ or $U2OS_{04:09N}$ transfected with the QYD- or KDV-minigene. (D) T_{QYD} were incubated with $U2OS_{04:09N}$ transfected with the QYD-minigene in the presence of a blocking antibody against pan HLA-class I (clone W6/32) or isotype control (clone MOPC 173). Bar charts indicate mean ± standard error of mean. In all charts, IFN-γ and granzyme B in cell culture supernatants were measured by ELISA. Data are representative of 3 independent experiments with 3 technical replicates per experiment. Statistical significance was determined by paired Student *t* test and corrected for multiple testing by Bonferroni correction. ***P<0.001.

B). Non-TCR-transduced T cells were not activated by QYD loaded onto PBMC of MM160, although granzyme B showed higher background after pre-incubation with IFN-γ (Figure 3B). To exclude the possibility that QYD could potentially be presented by any of the HLA-alleles of MM160 other than C*04:09N, we expressed all HLA-alleles of MM160 one at a time (Table 1) in HEK_{HLA-KO}, each in combination with the QYD minigene, and tested for activation of T_{QYD}. Only C*04:09N in combination with the QYD minigene activated T_{QYD} (Figure 3C). T cells that were used for TCR transduction did not express either C*04:01 or C*04:09N (Table 1); to experimentally exclude antigen presentation by T cells used for TCR transduction, we demonstrated that incubation of non-transduced T cells and T_{QYD} in presence of QYD peptide did not result in T-cell activation (Figure 3D).

In summary, PBMC of a C*04:09N$^+$ individual can present peptide and trigger T-cell activation in a C*04:09N-restricted manner.

Discussion

C*04:09N, first described in 2002,[5,7] is considered a null allele due to previously non-detectable cell surface expression, and we are not aware of any functional confirmation that C*04:09N could not trigger peptide-specific T-cell activation. Moreover, we recently identified a multiple myeloma-reactive TCR that appeared to recognize antigen in the context of C*04:09N.[8] This encouraged us to experimentally address whether C*04:09N expression: i) can be detected at the cell surface with current technologies; and ii) can trigger peptide-specific T-cell activation in a functional assay.

Accurate detection of C*04:09N cell surface expression requires specific antibodies, which are commercially available against pan HLA-class I (clone W6/32), HLA-BC (clone B1.23.2), and HLA-C (clone DT9). Currently, there is no commercially available antibody specifically against C*04:01, or even C*04:09N. Therefore, we generated two cell lines (HEK293T and U2OS), in which we deleted all endogenous HLA-A, HLA-B, and HLA-C alleles by CRISPR Cas9. In these cell lines, we expressed single HLA-C alleles of choice, which could subsequently be detected by any of the above-mentioned antibodies (pan HLA class I, HLA-BC, or HLA-C). We chose HEK293T and U2OS because they are widely available and easy to transfect with high efficiency by standard methodologies. The HLA-C-specific antibody (DT9) delivers only weak staining for flow cytometry compared to the HLA-BC or pan HLA-class I-specific antibodies; furthermore, DT9 can also recognize HLA E,[15] and was, therefore, not used in this study.

Recombinant cell surface expression of C*04:09N was weak and only reliably detectable with the HLA-BC-specific antibody (B1.23.2) confirming results of previous studies that only used the pan HLA-class I-specific antibody (W6/32) for C*04:09N detection.[5,7] A possible explanation, among many others, for the observed differences in sensitivity between the B1.23.2 and W6/32 antibodies could be that W6/32 detects HLA in complex with β2-microglobulin, whereas B1.23.2 recognizes an epitope expressed on both β2-microglobulin-associated and free HLA-class I heavy chains.[16,17]

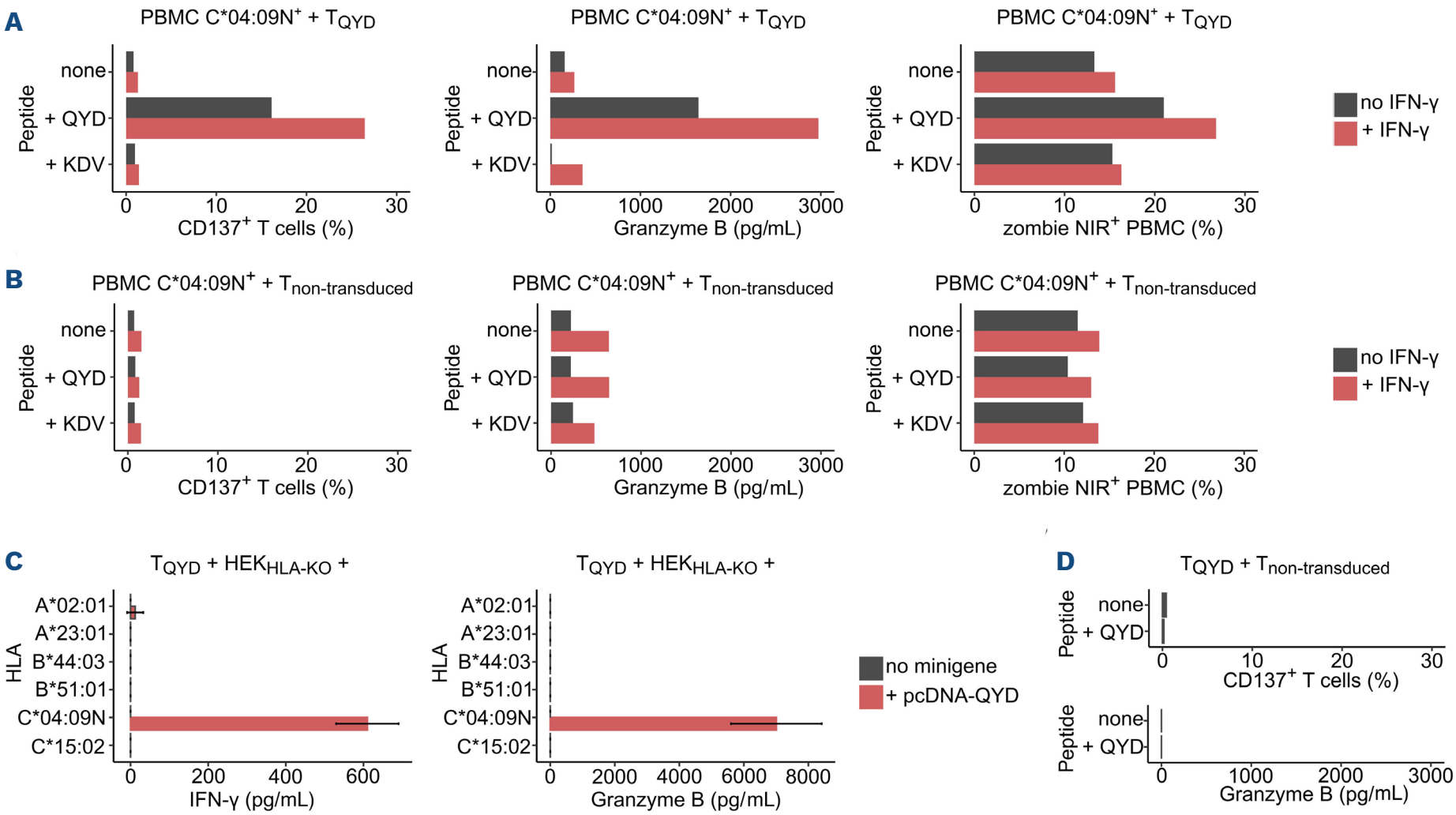

Figure 3. QYD peptide-specific T-cell activation in an HLA-C*04:09N+ individual. (A) T_{QYD} were labeled with cell trace violet and cultured with peripheral blood mononuclear cells (PBMC) of an HLA-C*04:09N+ individual loaded with 5 μM QYD- or KDV-peptide; PBMC were incubated with 1,000 U/mL IFN-γ for 24 hours (h) before co-culture if indicated. To detect T-cell activation, CD137 expression on TCR-transduced CD8+ T cells was determined by flow cytometry and granzyme B secretion in cell culture supernatants was measured by ELISA. Dead PBMC were determined as zombie NIR+ among all cell trace violet negative cells. (B) Non-TCR-transduced T cells labeled with cell trace violet and incubated with PBMC of the same donor (see panel A) loaded with 5 μM QYD- or KDV-peptide; PBMC were incubated with 1,000 U/mL IFN-γ for 24 h before co-culture if indicated. To detect T-cell activation, CD137 expression on CD8+ T cells was determined by flow cytometry and granzyme B secretion in cell culture supernatants was measured by ELISA. Dead PBMC were determined as zombie NIR+ among all cell trace violet negative cells. (C) T_{QYD} were cultured with HEK_{HLA-KO} that recombinantly expressed all HLA alleles of MM160 (one at a time) and the QYD-minigene. IFN-γ and granzyme B in cell-culture supernatants were measured by ELISA. Bar charts indicate mean ± standard error of mean. Data are representative of 3 experiments. (D) To confirm that QYD could not be presented by the T cells used for T-cell receptor (TCR) transduction, T_{QYD} were co-cultured with non-TCR-transduced T cells loaded with 5 μM QYD- or KDV-peptide.

C*04:09N surface expression was enhanced by IFN-γ exposure. IFN-γ is known to regulate gene expression beyond HLA-class I genes.[18-20] *In vitro* application of IFN-γ resulted in substantially increased C*04:09N expression and stronger specific T-cell activation. Based on these observations, we assume relevant effects of the cytokine milieu and tissue-associated modulation of C*04:09N expression *in vivo*. Independently of whether C*04:09N cell surface expression was detectable by flow cytometry, we asked experimentally whether C*04:09N expression was functionally relevant and could mediate peptide-specific T-cell activation. We demonstrated in two unrelated cell lines that transient or stable C*04:09N expression was sufficient for peptide presentation and specific T-cell activation. Inhibition of T-cell activation by addition of pan HLA-class I antibody to the cell culture indicated that TCR-peptide-HLA interaction occurred at the cell surface. Our results were confirmed with PBMC of a C*04:09N+ individual. Our data prove functionally that C*04:09N expression, albeit challenging to detect by flow cytometry, is biologically relevant for HLA-restricted T-cell activation.

Our study was entirely carried out *in vitro*; therefore, we cannot draw conclusions on *in vivo* situations. However, strong modulation of C*04:09N expression by the cytokine milieu (IFN-γ), and isolation of a clonally expanded C*04:09-restricted T-cell clone from bone marrow of a multiple myeloma patient[8] strongly suggest immunological relevance of this allele *in vivo*.

We assume our findings to have implications in the fields of hematopoietic stem cell and solid organ transplantation; however, although C*04:09N is considered one of the most abundant null alleles, its overall frequency is relatively low and varies within several orders of magnitude between different populations.[2] Comprehensive analyses of larger cohorts along with C*04:09N peptidome data, especially in comparison with C*04:01, are required to determine impli-

cations for allogeneic transplantation and specific disease associations, as well as in the context of other settings, such as cellular therapy.

Disclosures

LB has sat on advisory committees for Abbvie, Amgen, Astellas, Bristol-Myers Squibb, Celgene, Daiichi Sankyo, Gilead, Hexal, Janssen, Jazz Pharmaceuticals, Menarini, Novartis, Pfizer, Sanofi, and Seattle Genetics, and has received research support from Bayer and Jazz Pharmaceuticals. LH has sat on advisory committees for Sanofi, and has received travel support from Amgen. All other authors have no conflicts of interest to disclose.

Contributions

CW and LH conceived the project. CW, MLW, SS, JS, HH, AD, TB and LH designed experiments. AD and TB provided critical material. CW, MLW and SS performed experiments. CW, MLW, SS, LB, JS, HH, AD, TB and LH analyzed data. CW and LH wrote the manuscript with input from all authors. LH co-ordinated and supervised the project.

Acknowledgments

We thank Matthias Truss for flow cytometry support, and Klaus Dornmair for providing expression vectors.

Funding

This work was supported by Deutsche Krebshilfe e.V. (70113355; to LH), Berliner Krebsgesellschaft e.V. (HAFF202013 MM; to LH), the German Cancer Consortium (DKTK; to LH), and the European Union (ERC Advanced Grant Neo-T, 882963; toTB).

Data-sharing statement

All data are publicly available along with this manuscript.

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