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Wetering, R. van de; Kampstra, A.S.B.; Kester, M.G.D.; Tjokrodirijo, R.T.N.; Ru, A.H. de; Veelen, P.A. van; ... ; Toes, R.E.M.

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Targeting autoreactive B cells in rheumatoid arthritis through MHC class I-presented BCR-derived neo-epitopes

Renee van de Wetering [a,*], Arieke S.B. Kampstra [a], Michel G.D. Kester [b], Rayman T.N. Tjokrodirijo [c], Arnoud H. de Ru [c], P.A. van Veelen [c], Cynthia M. Fehres [a], Mirjam H.M. Heemskerk [b], René E.M. Toes [a]

[a] Department of Rheumatology, Leiden University Medical Center, Albinusdreef 2, Leiden, 2333 ZA, the Netherlands
[b] Department of Hematology, Leiden University Medical Center, Albinusdreef 2, Leiden, 2333 ZA, the Netherlands
[c] Center for Proteomics and Metabolomics, Leiden University Medical Center, Albinusdreef 2, Leiden, 2333 ZA, the Netherlands

A B S T R A C T

Somatic mutations can generate neoantigens recognized by $CD8^+$ T cells, enabling targeted elimination of mutated cells. Currently established B cell-targeting therapies in human B cell-mediated autoimmune diseases lack specificity and fail to achieve durable remission. Since autoreactive B cells often express B cell receptors (BCRs) that have undergone extensive somatic hypermutations (SHM), we hypothesized that mutated BCR-derived neoantigens could be exploited to selectively target these autoreactive B cells. However, the identification, validation, and extent to which autoreactive BCR-derived neoepitopes are presented and immunogenic remains poorly understood. Here, we investigated the immunological targetability of autoreactive B cells producing anti-citrullinated protein antibodies (ACPAs) - the hallmark antibody response in rheumatoid arthritis (RA). By using an integrated approach combining *in silico* MHC class I binding analysis, peptide-elution mass spectrometry, and $CD8^+$ T cell-activation assays, we studied 9 common HLA class I alleles and 150-patient derived BCRs. Our results predict that all autoreactive BCRs harbor sequences presentable by at least 3 HLA class I alleles. Notably, 85% of these sequences contain SHMs that introduce amino-acid changes compatible with candidate neoepitopes. Mass spectrometric analysis of HLA class I-eluted peptides from three BCR-transduced cell lines confirmed the presence of multiple predicted neoepitopes and revealed additional unpredicted, BCR-derived peptides. Importantly, several of these neoepitopes were endogenously processed, presented, and recognized by primary human $CD8^+$ T cells, while their unmutated counterparts were not recognized by the same $CD8^+$ T cell clones. These findings highlight that an integrative strategy, combining MHC isolation, peptide identification, and BCR sequencing, serves as a powerful platform for uncovering neoantigens presented by autoreactive B cells in a major human autoimmune disease. Likewise, they reveal a novel approach for the specific targeting of autoreactive B cells, paving the way for personalized immunotherapeutic interventions in RA and potentially other B cell-driven autoimmune diseases.

1. Introduction

Autoimmune diseases affect approximately 10% of individuals world-wide and are characterized by the breakdown of immune tolerance leading to tissue and organ damage [1]. Approximately 80% of affected individuals are female, and disease incidence increases with age. Despite substantial therapeutic advances over the past two decades, the majority of autoimmune diseases remain incurable, representing a significant public health and socioeconomic burden, as patients frequently experience severe morbidity necessitating lifelong pharmacological management.

B cell dysregulation represents a key pathological feature across numerous autoimmune diseases such as systemic lupus erythematosus, ANCA associated vasculitis, myasthenia gravis and rheumatoid arthritis (RA). This is substantiated by the presence of disease-specific autoantibodies, which often correlate with disease activity and progression. Despite divergent clinical manifestations in different autoimmune diseases, the shared B-cell mediated pathophysiology is supported clinically by the therapeutic success of B cell–targeting monoclonal antibodies in diverse autoimmune conditions [2], and more recently, by the remarkable, potentially curative responses to CD19-CAR T cell therapy in severe refractory autoimmune diseases [3–5]. Together, these observations indicate that the autoreactive B cell is central in driving pathogenesis observed in these autoimmune diseases.

B cells play pivotal roles in a healthy immune system through the ability to recognize and respond to a large diversity of pathogenic antigens, via their highly diverse B cell receptors (BCRs). Upon recognition of antigen, B cells become activated and start to secrete antibodies to neutralize and eliminate the targeted molecules/invading pathogens. During a normal B cell response, B cells diversify their BCRs through

* Corresponding author.
E-mail address: r.van_de_wetering@lumc.nl (R. van de Wetering).

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somatic hypermutation (SHM) [6]. This process creates unique BCR structures that can improve the affinity of the B cell for its target, allowing these B cells to outcompete others for antigen binding. In this way, SHM introduces unique sequence changes that are not encoded in the germline, potentially creating neoepitopes that the immune system has never encountered before and that may be recognized by $CD8^+$ T cells. At present, little is known about the possibility to employ $CD8^+$ T cells in the specific targeting of autoreactive B cells [7]. Nonetheless, $CD8^+$ T cell-based targeting of autoreactive B cells holds promise for highly selective depletion of autoreactive B cells throughout the body, extending to long-lived plasma cells that persist in protective niches such as the bone marrow. Compelling evidence that $CD8^+$ T cells can recognize and eradicate unwanted cells bearing somatic mutations comes from the tumor field. Both immune checkpoint blockade and neoepitope-based vaccination have shown that tumor-specific CD8 T cell responses can be mobilized against tumor-derived neo-epitopes, leading to eradication of established malignancies [8–10]. This concept suggests a parallel opportunity for eradication of established, somatically mutated B cell responses in autoimmune disease.

RA serves as a compelling model to explore this notion in more depth in the context of human autoimmune diseases. RA affects approximately 1% of the population and is driven by chronic synovial inflammation causing swollen, painful joints and bone erosions. One hallmark of RA is the presence of autoantibodies, anti-citrullinated protein antibodies (ACPA) directed against citrullinated(self)proteins [11]. ACPA-positivity is associated with a more severe disease course and B cell depleting therapies are highly effective in achieving clinical remission [12,13]. ACPA-expressing B cells undergo extensive SHM, with an average of 52 and 25 nucleotide mutations in the heavy and light chain respectively [14], translating to approximately 75% non-silent amino acid changes (unpublished data). Thus, ACPA-expressing B cells in RA are attractive candidates to investigate the feasibility of specific targeting of autoreactive B cells by $CD8^+$ T cells in the context of autoimmune disease, for example by vaccines expressing by BCR-derived neo-epitopes. In this study, we show MHC-I presentation of RA patient's $ACPA^+$ BCR-derived neo-epitopes and demonstrate the presence of $CD8^+$ T cells specific for these peptides in the circulation of HLA-matched healthy individuals.

2. Methods

2.1. Cell lines and primary material

Patient blood samples were taken upon obtaining written informed consent before inclusion and with approval from the ethical review board of the LUMC, The Netherlands (protocol P17.151). Buffy coats from healthy donors were obtained after written informed consent (Sanquin Blood Bank, Amsterdam, The Netherlands, protocol NVT0104.01).

K562 cells lacking HLA class I and class II surface expression were cultured in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Switzerland) with 10% fetal bovine serum (Thermo Fisher Scientific, USA), 200 mM L-glutamine (Lonza), and 10,000 U/ml penicillin/streptomycin (Lonza). Cells were transduced with HLA class I using the pLZRS vector encoding for HLA-A*02:01, HLA-A*03:01, HLA-A*24:02-IRES-mCD19, HLA-B*07:02, HLA-B*15:02-IRES-tNGFR, HLA-B*35:01-IRES_mCD19, and BCR sequences using the pMP71 vector with BCR_HC-IRES-BCR_LC-eGFP. Full-length BCR sequences from $ACPA^+$ patients with RA were obtained as described earlier [15–17]. B cell receptor germline sequences were determined using IMGT/V-QUEST [18]. The constructs were ligated into a pMP71 (+) expression vector carrying the IGHG1 and IGLC3/IGKC constant regions (UniProt). Ramos B cells without endogenous IGHM, IGHD and IGLC (MDL), and activation-induced cytidine deaminase (AID), were transduced with our BCRs of interest with similar expression vectors, but using a different plasmid backbone and retroviral system, as described here [16].

The generation of the immortalized RA1003.4 ACPA-expressing B cell line derived from an RA patient was reported previously [15], using a protocol described in more detail in an earlier publication [19]. In short, memory B cells were sorted and cultured in the presence of CD40-expressing L fibroblasts and recombinant mouse IL-21, and subsequently transduced with Bcl-6/Bcl-xL retroviral constructs by Phoenix-packaging cell-produced viral particles.

T cells isolated from healthy donors were cultured in T cell medium (TCM) consisting of IMDM with 5% fetal bovine serum, 5% human serum, 300 mM L-glutamine, 10,000 U/ml penicillin/streptomycin, and 100 IU/mL IL-2 (Novartis, Switzerland), and in the presence of pooled allogeneic PBMCs and EBV-transformed B cells (JY cell line) and 0.8ug/mL PHA.

2.2. Experimental HLA-peptidomics

Cell pellets were lysed in 50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetate, and 0.5% Zwittergent 3-12 (pH 8.0) supplemented with cOmplete Protease Inhibitor (Sigma-Aldrich). After 2 h of tumbling in lysis buffer at 4°C, cell lysates were centrifuged for 10 min at 1000 g at 4°C. The supernatant was subsequently centrifuged for 35 min at 13,000 g at 4°C, precleared with Protein A Sepharose CL-4B beads (GE Healthcare Life Sciences), and subjected to an immunoaffinity column with dimethyl pimelidate–immobilized W6/32 antibody (3 mg/ml resin) on Protein A Sepharose CL-4B beads with a flow rate of 1 ml/min. After washing with 5 to 10 column volumes of lysis buffers and 10 mM Tris-HCl (pH 8.0) buffers with 1 M, 120 mM, and no NaCl, bound HLA class I–peptide complexes were eluted from the column and dissociated with 3 to 4 column volumes of 10% acetic acid. Peptides were separated from HLA class I molecules via passage through a 10 kDa membrane (Macrosep Advance Centrifugal Devices With Supor Membrane, Pall Corp.), after which a further cleanup was done by solid phase extraction. Peptides were eluted in two fractions with 20 and 30% acetonitrile in 0.1% formic acid. The eluates were freeze dried.

Samples were dissolved in water/formic acid (100/0.1 v/v) and analyzed by on-line C18 nanoHPLC MS/MS with a system consisting of an Ultimate3000nano gradient HPLC system (Thermo, Bremen, Germany), and an Exploris480 mass spectrometer (Thermo). Fractions were injected onto a cartridge precolumn (300 μm × 5 mm, C18 PepMap, 5 μm, 100 A, and eluted via a homemade analytical nano-HPLC column (50 cm × 75 μm; Reprosil-Pur C18-AQ 1.9 μm, 120 A (Dr. Maisch, Ammerbuch, Germany). The gradient was run from 2% to 36% solvent B (20/80/0.1 water/acetonitrile/formic acid (FA) v/v) in 120 min at 250 nl/min. The nano-HPLC column was drawn to a tip of ~10 μm and acted as the electrospray needle of the MS source. The mass spectrometer was operated in data-dependent Top 20 MS/MS mode, with a HCD collision energy at 30% and recording of the MS2 spectrum in the orbitrap, with a quadrupole isolation width of 1.2 Da. In the master scan (MS1) the resolution was 60,000, the scan range 300-1500, at standard AGC target @maximum fill time of 'standard'. A lock mass correction on the background ion $m/z = 445.12003$ was used. Precursors were dynamically excluded after n = 1 with an exclusion duration of 10 s, and with a precursor range of 20 ppm. Charge states 1 (precursor selection range 800-1500) 2 (precursor selection range 400-800) and 3 (precursor selection range 300-600) were included. For MS2 the first mass was set to 110 Da, and the MS2 scan resolution was 30,000 at an AGC target of 'standard'@fill time of 'auto'. In a post-analysis process, raw data were first converted to peak lists using Proteome Discoverer version 2.5 (Thermo Scientific), and then submitted to the Uniprot Minimal Human database (20647 entries), supplemented with the with the project-specific database, using Mascot v. 2.2.07 (www.matrixscience.com) for peptide identification. Mascot searches were done with 10 ppm and 0.02 Da deviation for precursor and fragment mass, respectively, and no

enzyme was specified. Methionine oxidation and cysteinylation of cysteine were set as variable modifications. The false discovery rate was set <1% and, in addition, peptides with mascot ion scores <35 were generally discarded. Relevant candidate peptides were manually checked, and synthetic variants confirmed the identifications. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD073511 [20].

2.3. Peptides

Peptides were assigned to HLA class I (HLA-I) alleles expressed by cells of origin for which the peptides contain anchor residues for binding the respective HLA allele according to NetMHCpan 4.1. Peptides were synthesized at the LUMC Peptide and Tetramer Facility Immunology (PTFI). Mass spectra of identified peptides were compared to mass spectra of synthetically generated peptides to confirm correct identification.

2.4. pHLA tetramer-based T-cell isolations

PE- and APC-labeled pHLA tetramers were produced as previously described with minor modifications. PBMCs were isolated from complete buffy coats (Sanquin) by Ficoll-Paque gradient centrifugation. Buffycoats were obtained from healthy donors positive for one or more HLA-I alleles of interest to isolate BCR-derived peptide-specific T cell clones as previously described [21]. In short, PBMCs were incubated with pooled pHLA tetramers and pHLA tetramer bound cells were enriched by MACS using anti-PE MicroBeads (Miltenyi Biotec). These MACS-enriched fractions were stained with a LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific), as well as anti-CD4 BV605 (RPA-T4, BD Biosciences), anti-CD8 AF700 (RPA-T8, BD Biosciences), anti-CD14 BV510 (M5E2, Biolegend), anti-CD19 BV510 (SJ25C1, BD Biosciences). In the final experiments, APC-labeled pHLA tetramers were also added to the staining mix to sort the pHLA-double positive $CD8^+$ T cells only. pHLA-tetramer (double) positive $CD8^+$ T cells were single cells sorted into 96-well round-bottom plates containing feeder mix consisting of 5×10^5 irradiated PBMCs, 5×10^4 irradiated EBV-LCL JY cells and 0.8 mg/ml PHA in TCM. Every 3-4 days, the medium was replenished with fresh medium supplemented with IL-2 until IFN-γ-based screening for positivity was performed around day 13/14.

2.5. IFN-γ ELISA

T cell clones were tested for IFN-γ production 10-14 days after (re) stimulation by co-culturing 5.000 T cells per clone and 30.000 target cells in 30 μl T cell medium in 384-well plates. Target cells were either the K562 cells transduced with relevant HLA class I molecule only, K562 cells pulsed for at least 1.5 h at room temperature with various peptides of interest (in decreasing concentrations starting at 1 μM) and washed twice before co-culture, or with the BCR-transduced cell lines (K562 or Ramos) that endogenously process and present peptides. Phorbol-12-myrisitate 13-acetate (PMA) and ionomycin (both Sigma-Aldrich, dissolved in DMSO) were used as positive control for $CD8^+$ T cell stimulation in end-concentrations of 20 ng/ml and 1 μg/ml respectively. After 18 h, co-culture supernatant was used for IFN-γ detection in ELISA (DY285B, R&D Systems) according to the manufacturer's instructions.

2.6. Statistical analysis

If applicable, details are provided in the respective figure legends. GraphPad Prism 9.3.1 was used for statistics.

3. Results

3.1. The majority of ACPA BCRs is predicted to present neoepitopes in common HLA class I molecules

As HLA class I-restricted $CD8^+$ T cells are able to recognize and respond to neo-epitopes generated by somatic mutations [10], we first set out to determine whether the somatic mutations present in patient-derived autoreactive BCRs give rise to peptides with neoantigenic potential. Therefore, 150 sequences of BCRs from isolated citrullinated-antigen directed B cells of RA-patients obtained and used to assess whether peptide sequences from these autoreactive BCRs could be presented in common HLA-class I molecules [14]. To this end, we performed *in silico* peptide-binding predictions using NetMHCpan 4.1, focusing on nine HLA class I alleles prevalent across diverse ethnic backgrounds: HLA-A*01:01, HLA-A*02:01, HLA-A*03:01, HLA-A*11:01, HLA-A*24:02, HLA-B*07:02, HLA-B*08:01, HLA-B*15:02, and HLA-B*35:01 [22,23]. NETMHCpan 4.1 defines weak and strong HLA ligands as the top 0.5% (strong binder) and top 2% (weak binder) predicted affinities compared to a set of random natural peptides. For each HLA allele tested, the majority of BCR sequences (mean 93.7%; range: 79.5–100.0%) were predicted to yield at least one HLA ligand with weak or strong binding affinity (Fig. 1A). In fact, for most BCR sequences (60, 7%), peptides could be identified predicted to bind to each of the nine studied HLA alleles (Fig. 1B). Among those predicted MHC class I-binding peptides, 85.5% contained at least one somatic mutation when compared to their corresponding germline BCR sequence, thus qualifying as putative neoepitopes (Fig. 1C). While most of the predicted HLA-ligands are situated in framework 3, sequences from other regions of the BCR also contained peptides predicted to bind (Fig. 1D). Taken together, these prediction studies indicate that somatically mutated autoreactive BCRs could frequently give rise to peptides with high HLA class I binding potential, supporting their candidacy as $CD8^+$ T cell targets across a wide range of common HLA haplotypes.

3.2. BCR-derived neoepitopes are naturally processed and presented by HLA-I molecules in engineered K562 cells

As we wished to analyze whether the predicted epitopes are recognized by $CD8^+$ T cells, we considered it important to first verify whether such neoepitopes are, indeed, processed and presented by BCR-expressing cells (Fig. 2A). To this end, we generated K562 cell lines expressing HLA-A*02:01 and HLA-B07:02, both frequently expressed in the Dutch population. We next expressed mutated patient-derived ACPA sequences in these cells (2G9, 3F3 and 7E4; Supplementary Table 1). K562 cells are devoid of surface HLA Class I expression and therefore widely used to generate antigen presenting cell lines without the risk that results will be compromised by endogenous HLA class I molecules [24]. For each cell line, HLA class I molecules were immunoprecipitated from cell pellets of $1*10^9$ K562 cells after which MHC ligands were eluted for analysis by LC-MS/MS. The peptide repertoires displayed the expected length distributions and binding motifs for the MHC class I alleles examined [25]. By taking advantage of the distinct binding motifs of HLA-A*02:01 and HLA-B07:02, we identified 14 HLA-ligands; 9 with a unique peptide core and several length variants of those (Supplementary Table 2A). The identity of eluted peptides was verified by comparing the mass spectra of the eluted to their synthetically manufactured counterparts. Notably, comparison of the peptide-elution results with the NetMHC predictions revealed that a substantial number of eluted peptides was not predicted by NetMHC (Fig. 2B; 2C). These were in part length-variants of predicted peptides, sharing the same binding core, and in part peptides not selected to be in the top 2% highest affinities by the prediction algorithm and therefore initially not categorized as 'predicted' in our study (Fig. 2D). Importantly, all eluted and predicted peptides contained somatic hypermutations as compared to germline (Fig. 2D). Thus, these results show that the breadth of

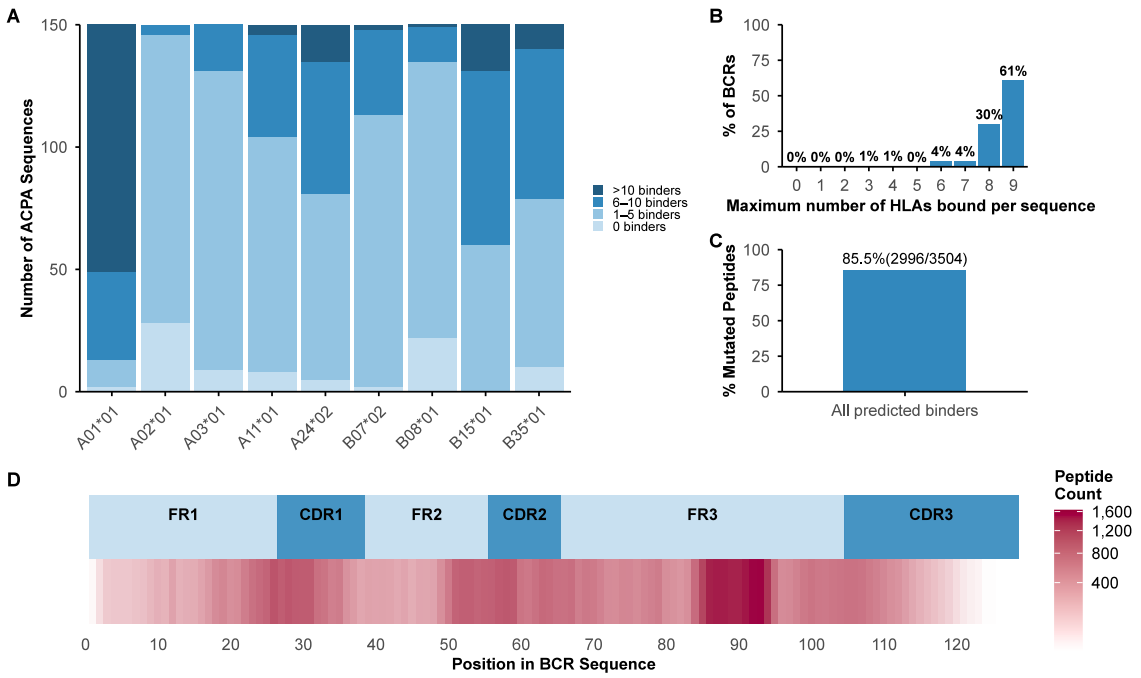

Fig. 1. Predicted binders for several common HLA alleles for 150 ACPA BCR sequences. Predicted 8–11mer peptides from 150 ACPA BCR heavy chain (HC) variable region sequences were evaluated for binding to nine common HLA class I alleles (HLA-A*01:01, A*02:01, A*03:01, A*11:01, A*24:01, HLA-B*07:02, HLA-B*08:01, HLA-B*15:02, and HLA-B*35:01) using NetMHCPan4.1. (A) Numbers of ACPA sequences containing predicted strong or weak binders for each HLA allele, categorized by the number of predicted binders per BCR sequence. (B) Distribution of the maximum number of HLA alleles bound by each of the 150 ACPA sequences shown in panel A. Each of the sequences is shown only in the bar according to the highest number of HLAs it binds. (C) Proportion of predicted peptides derived from the variable region containing ≥1 amino acid mutation compared with the corresponding IMGT/V-QUEST germline sequence. (D) Heatmap showing the number of predicted peptides in relation to the BCR sequence, including annotation of the framework (FR) and complementary-determining regions (CDR) regions.

BCR-derived peptide repertoire presented by HLA class I extends the repertoire predicted by a commonly used prediction algorithm and confirm that mutated BCR-derived neoepitopes can be presented by HLA class I molecules.

3.3. *BCR-derived neoepitope presentation is preserved in physiologically relevant B cell lines*

While K562 cells provide a well-established system to study MHC-I peptide presentation, they may not fully recapitulate the antigen processing and presentation environment of B cells [24]. To better approximate this native setting, we next examined primary ACPA-expressing B cell lines derived from two ACPA-positive RA-patients, immortalized via Bcl-6/Bcl-xL retroviral transduction [15]. Initial mass spectrometry analysis of a cell lysate of $1*10^9$ cells from one of these cell lines (RA1003.4) did not identify peptides derived from the BCR variable region (not shown). We then engineered K562 cell lines expressing combinations of the patient-derived BCRs together with relevant donor HLA Class I alleles (maximum of two alleles per cell line: HLA-A*03:01, HLA-A*11:01 & HLA-B*35:01 or HLA-A*24:02 & HLA-B*15:02). Across these cell lines, we identified 15 unique peptides corresponding to 11 distinct peptide cores (Supplementary Table 2C), including five peptides derived from the RA1003.4 BCR variable region. However, using a more sensitive parallel reaction monitoring (PRM)-based targeted MS using heavy-isotope-labeled peptide variants focusing on the five RA1003.4 BCR-derived peptides identified in the K562 system, we were still unable to detect those BCR neoepitopes in peptide elutions from the RA1003.4 immortalized B cell line.

Given the dependency of these immortalized cell lines on CD40L-expressing fibroblasts and IL-21 in culture, we next moved to a simpler, more controlled B cell model. We employed a variant of the human Burkitt lymphoma B cell line Ramos in which the endogenous IGHM, IGHD and IGLC (MDL) as well as activation-induced cytidine deaminase (AID) is knocked out, enabling expression of the same patient-derived 2G9, 3F3 and 7E4 ACPA-reactive BCRs previously assessed in the K562 model. We now focused on HLA-A*03:01, as the Ramos cell line expresses HLA-A*03:01 (biallelic), next to HLA-B*44:160Q, HLA-B*51:01 and HLA-C*16:01 (biallelic). Peptide elutions from the Ramos cell lines yielded a limited set of peptides: two from HLA-A*03:01, one from HLA-B*51:01 and two length-variants of the same peptide core for HLA-C*16:01 (Supplementary Table 2B). One additional peptide, SAADTAIYY, was eluted from the Ramos cells. While this peptide was predicted to have the highest binding affinity for HLA-C*16:01, *in silico* analysis also indicated potential binding to both HLA-B*51:01 and HLA-A*03:01, making its precise HLA-binding properties ambiguous. To complement this approach, we generated a K562 cell line expressing HLA-A*03:01 alongside the same three ACPA-derived BCR sequences. Elutions from this cell line yielded no additional peptides beyond those found in Ramos cells. In fact, of the two HLA-A*03:01-restricted peptides detected in Ramos cells, only one was also recovered from the K562-A*03:01 cells. This ligand was also the only HLA-A3*03:01 nonamer predicted using NetMHCPan4.1. Together, these results demonstrate that somatically mutated regions of autoreactive ACPA BCRs can be processed and presented by multiple class I HLA alleles in both K562 cells and B cells.

3.4. *BCR-derived neoepitope-reactive $CD8^+$ T cells are present in circulation of healthy individuals*

Next, we wished to investigate whether $CD8^+$ T cells are able to react to BCR-derived HLA-ligands. Therefore, we first generated pHLA monomers and then tetramers for cell sorting using HLA-A*02:01 and HLA- B*07:02 confirmed ligands from ACPA clones 2G9, 3F3 and 7E4. For all but 3 of the eluted peptides of interest, pHLA monomers could be refolded, further affirming the binding capacity to their respective HLA-I allele (Supplementary Table 3). All tetramers generated from these

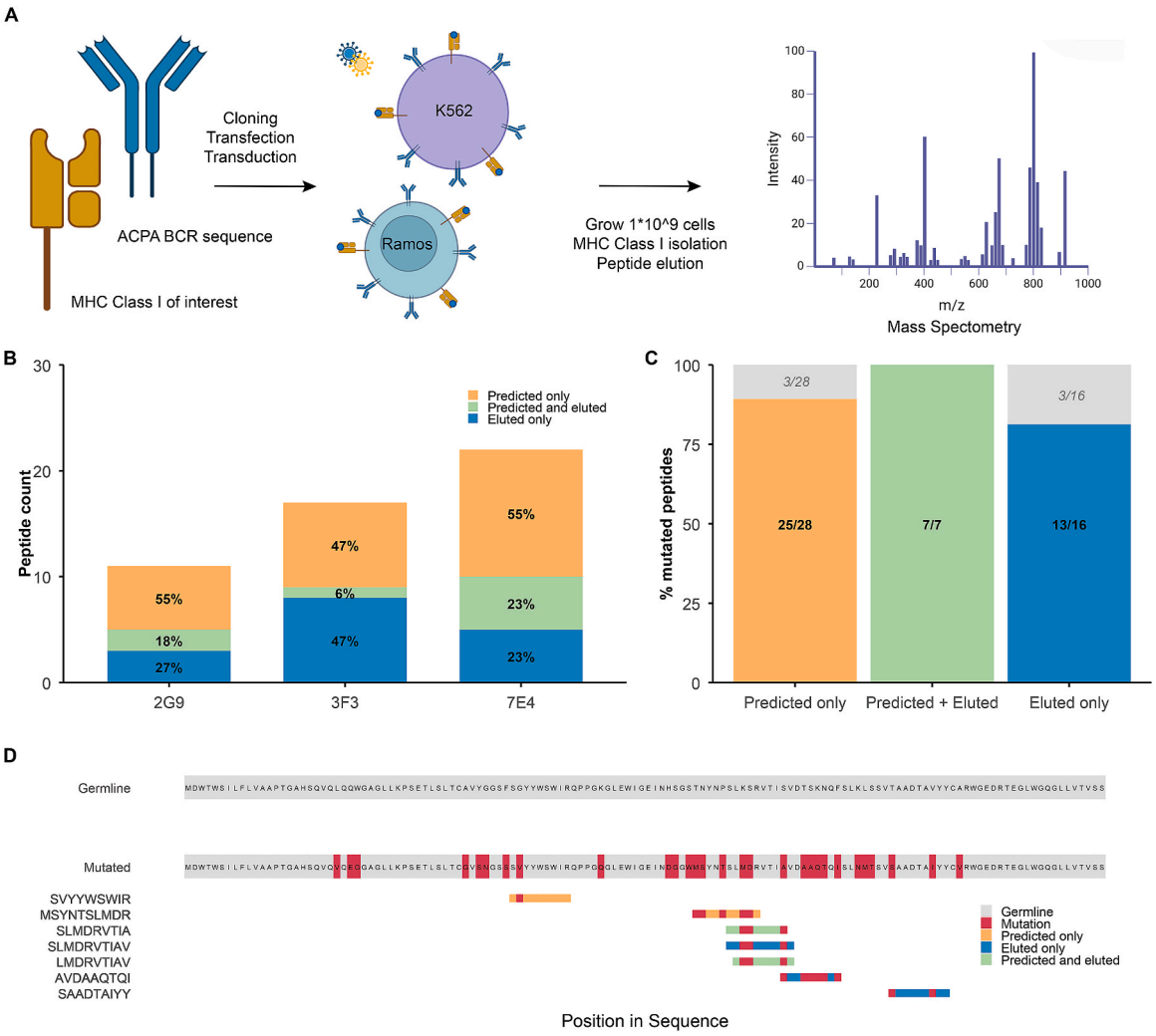

Fig. 2. Predicted versus eluted BCR-derived peptides. (A) Schematic representation of workflow. K562 cells were transduced with HLA-A*02:01, HLA-B*07:02, or HLA-A*03:01. Ramos cells contained endogenous HLA (HLA-A*03:01, HLA-B*44:160Q, HLA-B*51:01, HLA-C*16:01). Both were transduced with patient-derived autoreactive BCRs, 2G9, 3F3 or 7E4. (B) Peptide presentation from 2G9, 3F3, and 7E4 BCR sequences in K562 and Ramos cells. Peptides were categorized as 'predicted' if they were within the top 2% of predicted NetMHC affinities for that BCR sequence, compared to a set of random natural peptides. (C) Percentage and numbers of potential neo-epitopes (≥1 mutation compared to germline) in each peptide category (predicted, predicted and eluted and eluted only). Depicted in the grey part of the bar and italic text are the numbers of unmutated peptides per category. (D) Example of the 2G9 BCR variable domain sequence with all matching peptides; somatic hypermutations against the IMGT/V-QUEST germline reference are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pHLA monomers were used to stain and sort tetramer-positive CD8$^+$ T cells from HLA-matched healthy donor PBMCs.

In total, we sorted 1016 tetramer-positive CD8$^+$ T cells from fifteen donors positive for HLA-A*02:01 & HLA-B*07:02 on 4 different days and consecutively grew out these clones. Out of these, eighty-four CD8$^+$ T cells were reactive to at least one of the single peptides in screening experiments using respective HLA-expressing and peptide-loaded K562 cells. We found several CD8$^+$ T cell clones responsive to either one or both of the length variants of the (S)LMDRTIAV peptide (Fig. 3A). There were clear differences in the affinity of the T cell clones to their peptides (Fig. 3B). Crucially, although peptide-pulsing assays provide a robust and widely used approach to assess antigen specificity, they do not directly establish whether T cells detect antigen as naturally processed by their physiological target cells. Therefore, we next assessed whether the CD8$^+$ T cell clones responded to the ACPA-expressing engineered K562 cells. A subset of eight CD8$^+$ T cell clones derived from the naïve circulating repertoire from four donors produced IFN-γ upon co-culture with the BCR-expressing K562 cell lines (Fig. 3B; 3C). This demonstrates that these CD8$^+$ clones can recognize the naturally processed and presented BCR-derived neoepitope rather than only synthetic peptide provided exogenously.

To confirm the specificity of the CD8$^+$ T cell clones to the mutated neoepitopes only, we generated germline-reverted counterparts of the peptides. None of the CD8$^+$ T cell clones responded to the germline variants, supporting their selective recognition of the somatically mutated BCR-derived peptides (Fig. 3B; 3C). Taken together, these experiments demonstrate that CD8$^+$ T cells targeting BCR variable regions are present in the naïve CD8$^+$ T cell pool circulating in healthy donors and can be activated *in vitro* to specifically recognize endogenously processed and presented BCR-derived neo-epitopes.

3.5. CD8$^+$ T cell clones recognize BCR-derived neoepitopes in human Ramos B cells

To determine whether B cells would also be recognized by BCR-neoepitope-directed CD8$^+$ T cells, we next generated pHLA-A03:01 monomers and tetramers for the two HLA-A*03:01–restricted peptides identified in Ramos cells, as well as the HLA-ligand SAADTAIYY, which

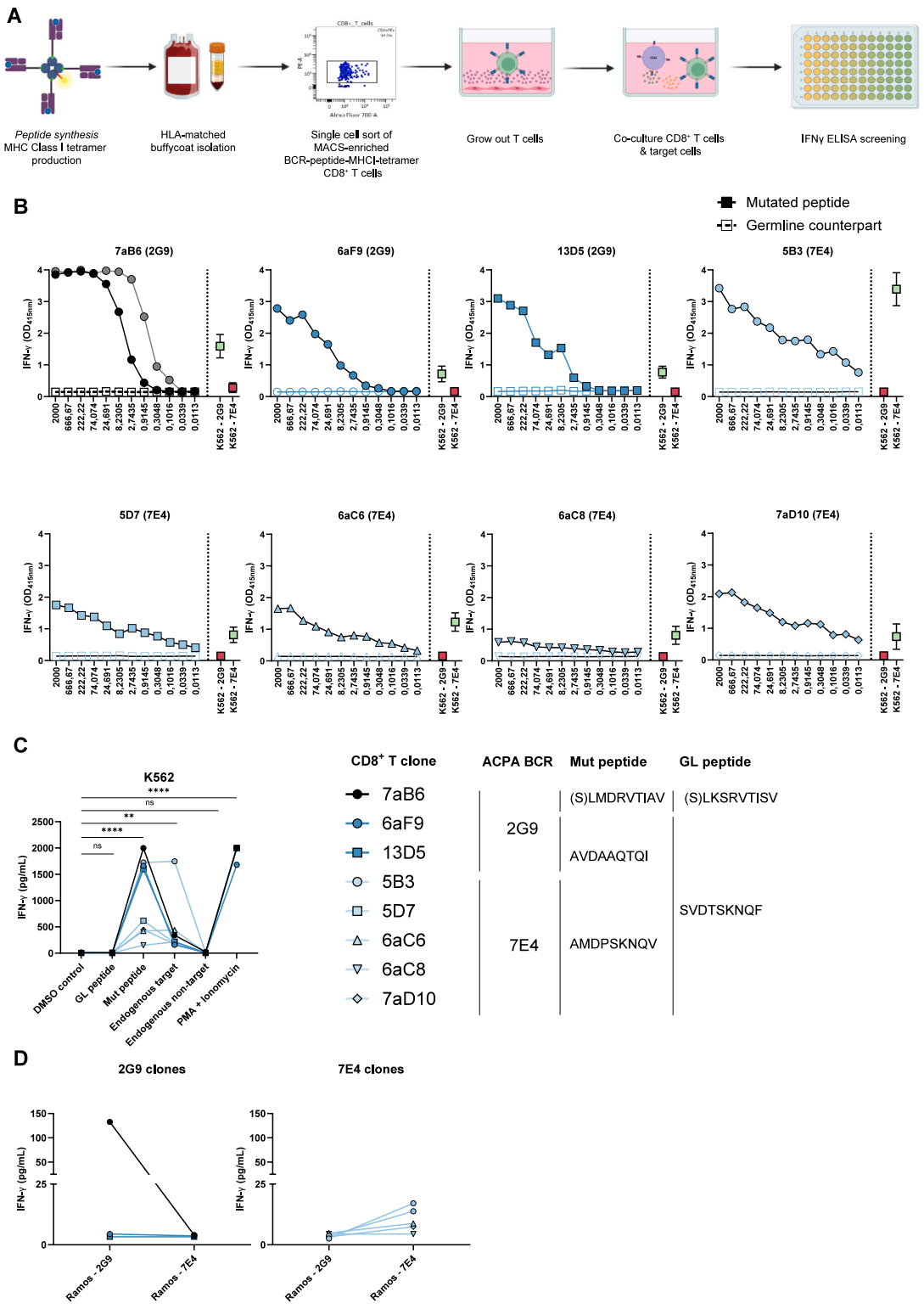

Fig. 3. CD8$^+$ T cell recognition of mutated and germline BCR-derived peptide sequences. (A) Experimental workflow to obtain BCR-peptide-reactive CD8$^+$ T cells. B) Representative IFN-γ ELISA results of eight CD8$^+$ T cell clones stimulated with K562 cells pulsed with either the BCR-derived peptide sequence (closed squares) or the germline-reverted variant (open squares) in titration. In each graph on the right are the results of the co-culture with K562 cells expressing the BCR containing the mutated peptide recognized by that CD8$^+$ T cell clone (green) or another BCR (red). Symbols and error bars represent mean and SD, respectively, of $n = 3$ experiments. (C) Summary of co-culture responses of the eight CD8$^+$ T cell clones shown in B with peptide-pulsed and BCR-transduced K562 cell lines, with IFN-γ measured by ELISA as readout. (S)LMDRVTIAV indicates both length variants of this peptide (SLMDRVTIAV and LMDRVTIAV). Bars and error bars represent mean and SD, respectively, of $n = 3$ experiments. One-way ANOVA with Dunnett's multiple comparison test was used to determine differences between stimulations. ns = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$. (D) Summary of IFN-γ ELISA results after co-culture of CD8$^+$ T cell clones with Ramos B cell lines transduced with HLA- A*02:01 and the 2G9 or 7E4 ACPA BCR sequence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

indeed formed a stable monomer with HLA-A*03:01. Although CD8$^+$ T cell clones could be isolated from four HLA-A*03:01–positive healthy donors, these clones showed only limited activation in response to peptide-pulsed target cells at higher peptide-concentrations and no detectable recognition of endogenously presented epitopes (data not shown). CD8$^+$ T cell reactivity to endogenously presented BCR-derived peptides by the Ramos B cell line's chromosomally encoded HLA molecules could therefore not be analyzed. We thus expanded our analysis of the Ramos cells to include previously validated HLA-A02:01-restricted CD8$^+$ T cell clones recognizing peptides from the 2G9 and 7E4 ACPA BCRs, by introducing HLA-A*02:01 into the 2G9 and 7E4 Ramos cell lines. In this setting, 4 out of 5 AMDPSKNQV-specific clones produced IFN-γ upon co-culture with HLA-A*02:01-transduced Ramos cells, and the highest-affinity clone (7aB6) robustly recognized the endogenously processed 2G9-derived neoepitope presented by Ramos cells (Fig. 3D). Together, these experiments demonstrate that rare but functional CD8$^+$ T cells specific for BCR-derived neoepitopes are present in healthy individuals and can be activated by BCR-neoepitopes that are endogenously processed and presented by human B cells.

4. Discussion

In this study, we investigated whether somatically mutated BCRs can serve as targets for CD8$^+$ T cell-mediated elimination of autoreactive B cells in autoimmune disease. Our findings provide multiple lines of evidence supporting this concept. We show *in silico* that numerous predicted BCR-derived neoepitopes can be presented across multiple common HLA alleles and experimentally confirm their presentation for several patient-derived ACPA-reactive BCRs. Importantly, our data also show that CD8$^+$ T cells that specifically recognized these mutated peptides can be activated from the naïve repertoire, indicating that they could also be induced *in vivo* by vaccination. In addition, our data indicate that these T cells can display sufficient functional avidity to detect endogenously processed neoepitopes on engineered (B) cell lines. Importantly, these T cells do not recognize germline counterparts, consistent with the absence of central tolerance to somatically introduced mutations. Together, the observations presented indicate that autoreactive BCR-specific CD8$^+$ T cells exist in the human repertoire and can be activated to recognize autoreactive B cells. Harnessing such responses in autoimmune patients could provide a highly selective strategy to deplete disease-driving B cells and reduce the long-term burden of autoimmune pathology and immunosuppressive therapy.

A next key question is whether B cells with BCR neoepitopes and CD8$^+$ T cells specific for those neoepitopes co-exists within the same individual. This question is challenging to approach in an *in vitro* setting as the frequencies of naïve T-cell clones with distinct specificities have been shown to be extremely low with for example certain CMV clones estimated at 1 in 33 million CD8$^+$ T cells in CMV-naïve individuals [26]. Because our data indicate that CD8$^+$ T cells specific for BCR-derived neoepitopes are as rare in the naïve repertoire, requiring a substantial volume of blood, we were not able to investigate their presence in RA patients in this study due to logistic and concomitant ethical restrictions. To show the presence of these cells in patients is relevant as it cannot be excluded that they might be rendered functionally unresponsive through tolerance mechanisms such as anergy or exhaustion. In this respect, it is possible that especially T cells expressing high affine anti-Ig TCRs are deleted or become anergic while low-to moderate affine TCRs recognizing these peptides remain present in the circulation. Likewise, one could speculate about other tolerance mechanisms at play in patient such as the presence of regulatory T cells specifically directed against neo-epitopes, common V-gene peptides or the constant regions of Ig-molecules [27].

Nonetheless, we also consider it more likely that BCR-neo-epitope-specific CD8$^+$ T-cells are present in patients – after having shown their presence in several donors- and that they can be activated by a vaccination approach similar to neo-epitope specific CD8$^+$ T cells in cancer. Because in it is known that in the latter condition, neo-epitope-specific CD8$^+$ T cells can be dormant for years, it is tempting to speculate that also BCR-neo-epitope-directed cells can be reactivated and expanded by vaccination, possible in combination with immune checkpoint inhibition therapy. The mechanisms governing central and peripheral T cell tolerance to the highly diverse BCR repertoire remain incompletely understood. The incomplete nature of tolerance to BCR-derived peptides is supported by prior work showing that B cells constitutively present BCR-derived peptides on MHC class II [28,29], combined with the observations that idiotype-specific CD4$^+$ T cell responses are present in mice [30]. Likewise, it has been observed that antibody maturation and somatic hypermutation are closely associated with the exclusion of MHC-II ligand content in antibody and BCR molecules, suggesting T cell-mediated removal of B cells [31]. Studies in the lymphoma field further directly show SHM-harbouring peptides presented in MHC Class I by lymphoma BCRs [32,33]. They also suggest CD8$^+$ T cell-mediated immunosurveillance of BCR sequences, with BCR sequences having lower immunogenicity in autologous compared to allogeneic HLA setting [34,35]. Taken together, these observations suggests that there is continuous T-cell mediated surveillance of all B cells and their BCRs, including but not restricted to autoreactive ones. Indeed, BCR-neoepitope-based vaccines can elicit both CD4$^+$ and CD8$^+$ T cell responses in lymphoma patients despite high expression of exhaustion markers on CD8$^+$ T cells at baseline [36,37]. However, these immunological responses did not always translate into clinical efficacy, possibly due to the tumour immunosuppressive environment, resulting in a downregulation and loss of MHC Class I expression on lymphoma B cells [38]. Logically, this would not directly apply in the context of autoimmunity [39]. Drawing parallels to successful cancer immunotherapy approaches, peptide vaccination aimed at targeting BCR-specific CD8$^+$ T cells may require combination with checkpoint inhibition to reverse tolerance and achieve functional activation [40].

Our results suggest that there is no B-cell-intrinsic barrier to presentation of autoreactive BCR-derived neo-epitopes and subsequent activation of neoepitope-specific CD8$^+$ T cells. Actually, native BCR expression in primary B cells can exceed B2M levels by up to 100-fold, far surpassing the approximately five-fold overexpression typical of our transduced K562 cell lines. While the HLA expression of individual alleles on primary B cells is conceivably lower than on the K562 cells, T cells are very sensitive in detecting HLA class I-restricted peptides compared to MS techniques and require only a few peptide – HLA complexes for activation [41]. Therefore, the number of potential neo-epitopes generated *in vivo* may be underestimated in our *in vitro* system of transduced BCRs.

Our *in vitro* system also has limitations. The CD8$^+$ T cell clones responded very differently to the two model cell lines used, K562 and Ramos. For example, the amount of IFN-γ produced by the CD8$^+$ T cell clone recognizing a peptide in the constant region of the BCR was much lower upon co-culture with the Ramos B cells compared to the K562 cells. Several factors may account for this discrepancy, including differences in processing of the transduced BCR, HLA expression and peptide loading efficiency, and specific interactions between both cell lines and the T cells - all of which may further differ in primary B cells. Indeed, for several CD8$^+$ T cell clones in earlier studies, we found much higher reactivity in co-cultures with primary tumorous material compared to K562 cells. In general, these and other factors (specifically protein abundance and processing [42,43] and HLA expression) could also explain the lack of peptide identification in the MHC class I peptidome of the Bcl-6/Bcl-xL-immortalized RA1003.4 ACPA-expressing B cell line, as well as discrepancies between peptides identified from K562 and Ramos cells each transduced with the same BCR. Although K562 cells are MHC class I deficient, they do express the processing machinery for MHC class I presentation, including transporter associated with processing (TAP) molecules [4]. As the transduced BCRs did contain a transmembrane domain and were expressed at the cell surface, we, therefore, consider it likely that to the trafficking and

antigen-processing routes are similar to an endogenously expressed membrane-bound BCR.

Finally, for therapeutic application, the size and breadth of the ACPA-expressing B cell repertoire will be an important determinant. A recent study using mass spectrometry analysis of unique antibody masses from RA patient serum, concluded that the ACPA antibody repertoire is highly diverse, although the top 10 clones can typically represent a substantial fraction of the repertoire [44]. While it is unclear how this secreted antibody pool directly relates to the cellular (memory) compartment [45], we observed a similarly oligoclonal repertoire at the B cell level, with evidence of partial clonal stability and recruitment of new clones over time [46]. This creates an opportunity for a focused, multiplexed vaccination strategy targeting neoepitopes from the most prevalent autoreactive clones. Targeting 10-15 dominant clones may eliminate a substantial proportion of autoreactive B cells in some individuals, potentially disrupting the self-perpetuating inflammatory cycles that characterize RA pathogenesis and having a significant impact on disease activity.

Taken together, these findings establish a novel paradigm for precision targeting of autoreactive B cells based on their somatically mutated BCR sequences. We have demonstrated that BCR-derived neoepitopes are processed and presented by HLA class I molecules and can be recognized by functional $CD8^+$ T cells, providing proof-of-concept for a fundamentally new therapeutic approach in autoimmune disease. The high mutation burden of ACPA-expressing B cells and broad predicted HLA presentability, suggests applicability to a large fraction of RA patients. Beyond RA, where we identified several potential bottlenecks including BCR repertoire breadth, this approach holds particular promise for autoimmune diseases characterized by more restricted BCR repertoires, such as in Vaccine-induced immune thrombotic thrombocytopenia (VITT) or MS [47,48]. Overall, our work provides both the conceptual framework and methodological platform for developing personalized, neoepitope-based therapies that could selectively eliminate autoreactive B cells in humans while preserving the broader protective immune repertoire.

CRediT authorship contribution statement

Renee van de Wetering: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Arieke S.B. Kampstra:** Investigation, Methodology, Writing – review & editing. **Michel G.D. Kester:** Methodology, Resources, Writing – review & editing. **Rayman T.N. Tjokrodirijo:** Formal analysis, Investigation, Writing – review & editing. **Arnoud H. de Ru:** Formal analysis, Investigation, Writing – review & editing. **P.A. van Veelen:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Cynthia M. Fehres:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Mirjam H.M. Heemskerk:** Conceptualization, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **René E.M. Toes:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

n/a.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2026.103581.

Data availability

The mass spectrometry proteomics data are available in the PRIDE partner repository (see methods). All further data that support the findings of this study are available upon reasonable request.

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