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OPEN Single-hit genome editing optimized for maturation in B cells redirects their specificity toward tumor antigens

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T-cell-based adoptive immunotherapy is a new pillar of cancer care. Tumor-redirection B cells could also contribute to therapy if their manipulation to rewire immunoglobulin (Ig) genes is mastered. We designed a single-chain Ig-encoding cassette ("scFull-Ig") that redirects antigen specificity when inserted at a single position of the IgH locus. This design, which places combined IgH and IgL variable genes downstream of a pVH promoter, nevertheless preserves all Ig functional domains and the intrinsic mechanisms that regulate expression from the IgM B cell receptor (BCR) expression to Ig secretion, somatic hypermutation and class switching. This single-locus editing provides an efficient and safe strategy to both disrupt endogenous Ig expression and encode a new Ig paratope. As a proof of concept, the functionality of scFull BCR and/or secreted Ig was validated against two different classical human tumor antigens, HER2 and hCD20. Once validated in cell lines, the strategy was extended to primary B cells, confirming the successful engineering of BCR and Ig expression and the ability of scFull-Ig to undergo further class switching. These results further pave the way for future B cell-based adoptive immunotherapy and strategies to express a therapeutic mAb with a variety of switched H-chains that provide complementary functions.

Effective monoclonal antibodies (mAbs) are available for the treatment of many chronic diseases, particularly cancer. The high specificity of such biotherapies hereby limits their side effects compared to those of conventional treatments such as cancer chemotherapy. However, mAbs can in some cases lead to poor responses or resistance, particularly in patients with the lowest serum trough concentrations¹. Repeated injections of mAbs are then required to prevent relapses, which can be lengthy and costly, limiting the efficacy of these treatments. This is especially true for mAbs with rapid serum clearance, such as panitumumab or cetuximab, or for some bispecific antibodies¹. Strategies that could lead to continuous infusion of such antibodies, for example, as endogenously produced by the patient's own cells, would therefore represent a strong asset. The clinical use of some antibodies of high therapeutic interest but with chemical instability or manufacturing contingencies notably associated with some Ig classes could also be rescued by direct in vivo production in the patient's body. This strategy could also be relevant for conferring long-term immunity to some immunodeficient patients with a preserved B-cell pool but poor response to classical vaccination.

B lineage cells are the most suitable cell type for the correct folding and secretion of immunoglobulins (Igs) according to their different membrane-bound or secreted formats². At the plasma cell stage, these cells are capable of secreting large amounts of soluble antibodies, while memory B cells can also establish immune memory and respond to antigen (Ag) rechallenge, thereby contributing to protection against recurrent infections. B cells edited for Ig expression could therefore potentially endow patients with both aspects of a long-term immune response to a specific Ag. Several recent studies have shown that CRISPR-mediated editing of the B-cell genome is feasible in primary B cells and could reformat Ig variable (V) regions on purpose^{3–5}. This has been achieved by various strategies, in particular with the aim of establishing adoptive anti-HIV immunity, either by concomitantly engineering the genes encoding the Ig heavy (IgH) and light (IgL) chains, or by inserting cassettes

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including a complete IgL chain sequence upstream of an IgH VDJ sequence into the IgH locus^{6–8}, or by simply restricting the gene edition to the endogenous VDJ CDR3 to produce anti-HIV antibodies whose specificity is mainly IgH dependent^{9,10}. In the context of cancer immunotherapy and learning from the success of CAR-T cells, which have now achieved complete remission in many patients and have demonstrated the ability to safely and durably integrate into patients' immune system^{11–13}, further exploration and optimization of the conditions that might allow successful adoptive cell therapy based on B cells are warranted. In such a strategy, B cells taken from an individual are genetically modified to produce Ig with the desired specificity and then injected back into the original donor. Successful engraftment of engineered B cells would then be expected to result in long-term stable infusion of a therapeutic mAb into body fluids and/or artificially established immune memory against a tumor Ag.

For this purpose, we used CRISPR-mediated gene knock-in (KI), the best tool for precise gene editing^{14,15}, to hijack the inherent ability of B cells to produce Ig molecules. The insertion site for the KI was chosen within the Ig heavy chain (IgH) locus to simultaneously disrupt endogenous IgH synthesis and integrate the KI cassette in a position that mimics all the normal features of Ig expression. These include B lineage-specific transcription, upregulated transcription in activated B cells and plasma cells, regulation of alternative splicing between membrane and secreted type transcripts, and the ability to undergo class switch recombination (CSR) of the Ig constant(C) regions upon recombination between the endogenous S μ region and a downstream S region of the IgH locus.

Since in vitro cell culture and maintenance of B cells, as well as their genetic engineering, remain challenging, we have optimized B-cell culture conditions and CRISPR-mediated gene editing and now report an efficient strategy for gene editing of human primary B cells to produce a complete Ig of a given specificity in a single editing step. The genetic engineering of Ig theoretically requires the modification of two loci, one for the heavy (H) and one for the light (L) chains. To express the desired Ig and to disrupt endogenous Ig production after a single gene modification, we developed a new single-chain format of a whole Ig, “scFull-Ig”. This format resolves any issue about the need for stoichiometric expression of both Ig chains and precludes any mispairing with an endogenous L chain. Moreover, this approach has the advantage of positioning both the V_HDJ_H and V_LJ_L regions immediately downstream of the V_H promoter in the optimal position accessible to somatic hypermutation (SHM), with C regions downstream. Using this strategy, modified primary B cells were shown to be able to successfully express BCR membrane molecules in the scFull-Ig-Upstream Switchable Module (USM) format, thereby acquiring the corresponding Ag-binding activity and secreting scFull antibodies.

Materials and methods

Recombinant scFull-Ig production

To overcome the challenges associated with the expression of recombinant polymeric molecules, we designed a single-chain strategy to produce full-length Ig molecules by using two linker (Lnk) sequences to assemble the Ig domains in the following order: [V_H-Lnk-V_LC_L-Lnk-C_{H1}]. We designed different expression vectors and gene cassettes first to validate the functionality of the expected soluble antibodies (anti-HER2 scFull-Ig and anti-hCD20 scFull-Ig) and second to edit the IgH locus in the B-cell genome to encode Ig in this scFull format (after integration of an “scFull-Ig USM” gene cassette).

All the vectors were assembled using the NEBuilder HiFi DNA Assembly strategy (New England Biolabs, Ipswich, MA) and combined with DNA fragments either PCR-amplified from human genomic DNA or custom synthesized on purpose (Genecust, Boynes, France).

The first aim was to validate the soluble form of sc-Full-Ig. Two vectors were assembled in the pcDNA3 expression vector backbone, which encoded the IgG1-class soluble forms of either the anti-HER2 scFull-Ig or the anti-hCD20 scFull-Ig, each following a [V_H-Lnk-V_LC_L-Lnk- γ 1(C_{H1}-C_{H2}-C_{H3})] architecture. These vectors are driven by a CMV promoter and then comprise, in a 5' to 3' order, a V_H leader exon, a V_HDJ_H exon, a first linker [(G4S)3-SG3S], the V_LJ_L and C_L exons, a second linker [S-(G4S)3] and, ultimately, the C_{H1}-C_{H2}-C_{H3} C exons of the γ 1 gene (followed by a polyadenylation site). Both linker sequences include the classical (G4S)3 sequence previously reported in a scFV design¹⁶. Both secreted antibodies were then produced by transfecting nonlymphoid cells, such as the XTEN-CHO cell line (ProteoGenix, Schiltigheim, France).

In a second aim, vectors were built to yield Ig with the same [V_H-Lnk-V_LC_L-Lnk] architecture after the IgH locus was modified. This was accomplished by precisely inserting into the IgH locus a cassette designed to be expressed in B cells and spliced to the endogenous IgH C genes. This gene cassette started with a B-cell-specific pV_H promoter (a 180 bp fragment from the mouse VH4.1 gene; NCBI reference NC000078.7) and ended with a donor splice site sequence identical to that of a J_H element. This [pV_H-V_H-Lnk-V_LC_L-Lnk USM] cassette was additionally flanked with 5' and 3' homology arms to promote homology-directed recombination (HDR) and precise insertion into the intron downstream of JH6. Once properly inserted, it could thus be expressed in association with the endogenous C_H genes from the targeted IgH locus.

Western blot

After reduction with NuPAGE Sample Reducing Agent (Invitrogen), scFull-Ig molecules were analyzed via Western blotting together with commercial rituximab (MabThera, Roche) and pertuzumab (Perjeta, Roche) as controls. After electrophoresis on 4–12% Bis-Tris 1.5 mm gels (Invitrogen), the proteins were transferred to nitrocellulose membranes using iBlot2 NC Mini Stacks and an iBlot2 Transfer Device (Invitrogen). The membranes were stained with 400 μ g/ml goat anti-human κ chain antibody (Southern Biotech), 0.2 μ g/ml donkey anti-goat IgG H+L HRP conjugated antibody (Bethyl Laboratories), or 1/10,000 diluted goat anti-human IgG HRP conjugated antibody (Sigma). Staining was detected using Pierce ECL Plus Western Blotting Substrate (Thermo Scientific).

Antibody-dependent and complement-dependent cytotoxicity assays

ADCC assays were performed using CD16-expressing reporter cells from the Jurkat-Lucia NFAT-CD16 cell line (InvivoGen) according to the manufacturer's protocol. Briefly, target cells were incubated with antibodies for one hour, after which Jurkat-Lucia NFAT-CD16 cells were added. After incubating for 6 h, the supernatant was transferred to a white microplate, and QUANTI-Luc solution (InvivoGen) was added. The light signal indicating Jurkat cell activation was quantified using a Synergy H1 luminometer (Agilent).

SUDHL4 target cells were incubated with antibodies for one hour, followed by the addition of rabbit complement (Cedarlane). After incubating for 45 min, the cells were washed, and viability was assessed via DAPI staining.

BCR signaling assays

The ability of the engineered BCR to trigger B-cell activation was demonstrated using phospho-flow assays, which monitor the intracellular phosphorylation of elements of the BCR signaling cascade (SYK, BLNK, PLC γ 2, pERK1 and BTK).

DG75 cells clonally expressing a scFull-Ig BCR were starved for 1 h with RPMI 1640 GlutaMAX medium (Gibco) supplemented with 1% fetal bovine serum (FBS). A stimulation step was then performed by adding the F(ab')₂ fragment goat anti-human Ig (anti-IgA + IgG + IgM, H + L) (Jackson ImmunoResearch) at 20 μ g/ml. The cells were then permeabilized and stained with appropriate antibodies specific for the phosphorylated forms of SYK, BLNK, PLC γ 2, BTK, and pERK1 (BD PhosFL) according to the supplier's protocol. The stained cells were analyzed on a CytoFlex flow cytometer (Beckman Coulter).

Cell culture

DG75 cells were grown in RPMI 1640 GlutaMAX medium (Gibco) supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco) and penicillin-streptomycin-glutamine (PS-G; 1 unit/ml penicillin, 1 μ g/ml streptomycin, and 2.92 μ g/ml L-glutamine; Gibco).

MCF7 cells were grown in DMEM (Gibco) supplemented with 10% FBS (Gibco) and PS-G.

SUDHL4 and hCD20^{+/−} EL4 cells were grown in RPMI 1640 GlutaMAX medium supplemented with 10% FBS (Gibco), 1 mM sodium pyruvate and PS-G. BT474 cells were grown in RPMI 1640 GlutaMAX medium supplemented with 10% FBS, NEAA (Gibco), 10 mM HEPES (Gibco), 0.45% D-glucose (Gibco), 1 mM sodium pyruvate and 10 μ g/ml bovine insulin (Sigma). SKBR3 cells were grown in RPMI 1640 GlutaMAX medium supplemented with 10% FBS, NEAA and 10 mM HEPES.

HEK293T cells were grown in DMEM supplemented with 10% FBS and 200 μ g/ml G418 (Gibco). Transfections of HER293T cells were performed with MacsFectin reagent according to the manufacturer's protocol.

Primary B-cell isolation and culture

Buffy coats from healthy volunteers (with informed consent) were obtained from the Etablissement Français du Sang (Rennes, France). B cells were isolated using the StraightFrom™ Buffy Coat CD19 Microbead Kit (Milenyi Biotec) according to the manufacturer's protocol. B cells were then cultured in RPMI 1640 GlutaMAX medium (Gibco) supplemented with 10% FBS (Gibco) and 1 mM sodium pyruvate (Gibco). B cells (0.75 $\times 10^6$ cells/ml) were stimulated for 4 days with the "1st step stimulation cocktail", which included 1 μ g/ml CpG oligodeoxyribonucleotide (CpG 2006; Miltenyi Biotec), 2.4 μ g/ml F(ab')₂ fragment goat anti-human IgA + IgG + IgM (H + L) (Jackson ImmunoResearch), 50 U/ml recombinant IL-2 (R&D Systems) and 100 ng/ml recombinant human soluble CD40L (Immunex). At day 2, 5 ng/ml IL-10 (R&D Systems) was added. At day 4, the activated B cells were washed and transferred at 0.5 $\times 10^6$ cells/ml into a "2nd step plasmablast differentiation cocktail"¹⁷ containing 50 U/ml IL-2 (R&D Systems), 5 ng/ml IL-4 (R&D Systems) and 12 ng/ml IL-10 (R&D Systems). To obtain plasma cells, the cells were washed at day 7 and cultured until day 10 at 0.5 $\times 10^6$ cells/ml with 50 U/ml IL-2 (R&D Systems), 250 U/ml IFN- α (R&D Systems), 20 ng/ml IL-6 (PeproTech) or 12 ng/ml IL-10 (R&D Systems).

Donor DNA preparation and gene editing

DNA templates for CRISPR-mediated KI were constructed from PCR-amplified fragments using the NEBuilder HIFI assembly kit (New England Biolabs). Donor DNA for CRISPR-mediated KI was prepared from plasmids by PCR amplification. PCR products were purified using a NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). The purified DNA was then ethanol precipitated and resuspended in H₂O to a final target concentration of 2000–5000 ng/ μ l, as quantified by Qubit. For CRISPR/Cas9, the IgH locus between JH6 and the E μ enhancer was targeted at a location chosen more than 0.4 kb downstream of JH6 to ensure sufficient 5' homology for introducing a transgene, irrespective of the preexisting endogenous VDJ rearrangement. After preliminary trials with less efficient targeted sites (not shown), we focused our experiments on the best target in our hands, with the sequence GGAAAGAGAACTGTCGGAGT (human genome GRCh38, Chr14, 105862762–105862781).

For the editing of B cell lines, one day prior to the CRISPR experiments, the cells were seeded at 0.2 $\times 10^6$ cells/ml to be in the exponential growth phase. The cells were electroporated using a Nucleofector Kit V (Lonza) as follows: 1.10⁶–3.10⁶ cells were centrifuged and resuspended in 100 μ l of supplemented Nucleofector solution. The RNP complex was formed by incubating 100 pmol of Alt-RTM S.p. HiFi Cas9 Nuclease V3 (IDT) with 500 pmol of sgRNA (Synthego) for 15 min at 37 °C. RNP was then added to the cell suspension, and the cells were transfected using the Nucleofector I program "X-01". Immediately after transfection, 900 μ l of culture medium was added, and the cells were allowed to rest for 5–10 min before being transferred to 6-well plates in 5 ml of medium.

Primary B cells were electroporated using an ATx device (MaxCyte). A total of 3.10⁶ cells were centrifuged, washed and resuspended in 25 μ l of electroporation buffer (MaxCyte). The RNP complex was formed by

Results

The scFull-Ig mAb format can be functional

We investigated a new single-chain format of Ig, the scFull-Ig (Fig. 1A), in which a first linker connects the H chain V domain to the κ L chain V and C domains, followed by a second linker and the full C region of the IgG H chain. To evaluate this format in the context of different associated V regions, we produced two different scFull-Igs, one against HER2 and one against hCD20, based on V sequences from pertuzumab and rituximab, respectively. Single-chain versions of both antibodies were first produced in serum-free media in XTEN-CHO cells transfected with expression vectors encoding, in each case, the linked V_HDJ_H region + complete κ L chain and second linker ([VDJ-Lnk-V κ] κ -C κ -Lnk] cassette), followed by the C domains of a human secreted $\gamma 1$ H chain. The structure of the secreted antibodies was investigated by western blotting using anti-hIgG and anti- κ antibodies after gel electrophoresis. Under reducing conditions, the scFull-Ig complex consisted of a single 75 kDa protein, as expected, that combines the Ig H and L chains (Fig. 1B).

We then validated the binding of such soluble scFull-Igs to their respective targets, as specifically expressed by the RAMOS, RAJI and hCD20⁺ EL4 cell lines for the anti-hCD20 scFull-Ig and MCF7, SKBR3 and MCF7 cells for the anti-HER2 scFull-Ig (Fig. 1C / Suppl. Figure 1). For the anti-HER2 mAbs, the staining results were quite similar for the classical format and the scFull-Ig format. Both anti-CD20 mAbs also specifically stained their targets, but the mean fluorescence intensity of the cells stained with the scFull-Ig was somewhat weaker than for those stained with the commercial rituximab. This finding suggested preserved specificity but some loss of affinity after the single-chain transformation of this antibody, as has been observed for other scFv derivatives of rituximab²¹.

The functionality of the scFull-Ig Abs was also validated by assessing their ability to mediate cytotoxicity toward appropriate target cells. Similar to rituximab, anti-hCD20 scFull-Ig was found to induce complement-dependent cytotoxicity (CDC) in a dose-dependent manner, but with a shifted curve requiring approximately twice the concentration of the antibody to achieve the same cytotoxicity (Fig. 1D). Like with rituximab and pertuzumab, anti-hCD20 scFull-IgG and anti-HER2 scFull-IgG specifically induced ADCC in cells harboring the target antigen (i.e. RAJI and MCF7 cells, respectively), even when used at low doses in the range of 3.1 ng/ml (Fig. 1E). Interestingly, we observed better induction of ADCC with the anti-HER2 agent scFull-Ig than with the commercial pertuzumab at an equivalent dose.

Taken together, these data confirm that the scFull-Ig format is suitable for the production of Ag-specific antibodies. Given these results and the notable efficacy of the anti-HER2 scFull-Ig format, this model was selected for subsequent gene editing experiments.

Knock-in of a spliceable single scFull-Ig upstream switchable module (scFull-Ig USM) cassette allows functional BCR expression in a B lymphocyte cell line and disrupts endogenous Ig expression

In addition to the advantages of a single chain to ensure stoichiometry and physical association of the paired H/L structure, the scFull format was designed to allow recombinant Ig production through a single CRISPR-mediated genomic edit at the IgH locus with a cassette that places both the VH and VL genes in an upstream position. Accordingly, an editing site was chosen downstream of the J_H region and upstream of the μ enhancer (Fig. 2A), as this position can allow the expression of a knock-in gene while concomitantly disrupting the expression of the endogenous IgH VDJ gene, which is thereby dissociated from μ . We preliminarily validated the high accessibility of this genomic site for CRISPR editing in the DG75 lymphoma cell line using an HDR template containing a pVH-promoter-tdTomato reporter cassette (Suppl. Figure 2A). This experiment also validated the efficacy of 500 bp homology arms, which were ultimately designed with terminal truncated Cas9 target sequences (tCTS) at both ends of the HDR template to optimize HDR²². Overall, this resulted in regular expression, as indicated by more than 30% fluorescent cells (Suppl. Figure 2B).

Based on this result, the anti-HER2 scFull-Ig USM cassette, preceded by a VH promoter, was flanked by the above validated HDR flanking arms to be inserted at the same genomic location downstream of J_H . For proper splicing to endogenous IgH C genes, the scFull-Ig USM gene cassette was then terminated with a J_H splice donor site (J_H 3'splice) downstream of the second linker. With such a complete [pVH-VDJ-Lnk-V κ] κ -C κ -Lnk J_H 3'splice configuration, this cassette can be expressed and spliced to the immediately downstream C gene. It then replaces the endogenous V_HDJ_H exon in IgH transcripts (Fig. 2A). A KI at this position, which respects the classical architecture of an IgH functional gene, is expected to preserve the process of alternate splicing of membrane-type vs. secreted-type IgH C transcripts. It also does not affect any of the elements that control the process of CSR at the locus. In cells undergoing CSR and/or plasma cell differentiation, such an edited IgH locus is therefore expected to behave as a normal endogenous IgH locus and to potentially be expressed under several parallel or successive clonally related BCR and secreted Ig forms from the various Ig classes (but sharing the same clonotypic Ag specificity).

To confirm these expectations, we first inserted the anti-HER2 scFull-Ig USM cassette into DG75 lymphoma cells with a mature B lymphocyte phenotype and IgM class BCR expression. This experiment assessed the ability of the membrane-bound scFull-IgM USM to bind soluble HER2 after CRISPR-mediated insertion of the [VDJ]_{pertu}-Lnk-V κ _{pertu}-C κ -Lnk- J_H 3'splice gene cassette (Fig. 2B). More efficient insertions were subsequently achieved by terminating HDR arms with tCTS sequences, which bind a noncleaving complex of specific gRNA and Cas9. Altogether, in DG75, approximately 37% of the cells were successfully edited and acquired the ability to bind soluble HER2, similar to the results obtained for the insertion of the tdTomato reporter cassette.

Validation of the on-target genomic integration of scFull-Ig USM into the IgH locus was based on a multiplex PCR assay (competitively involving 2 primers that flank the insertion site in the IgH locus and 1 primer that hybridized to the inserted scFull-Ig USM cassette) (Fig. 2C).

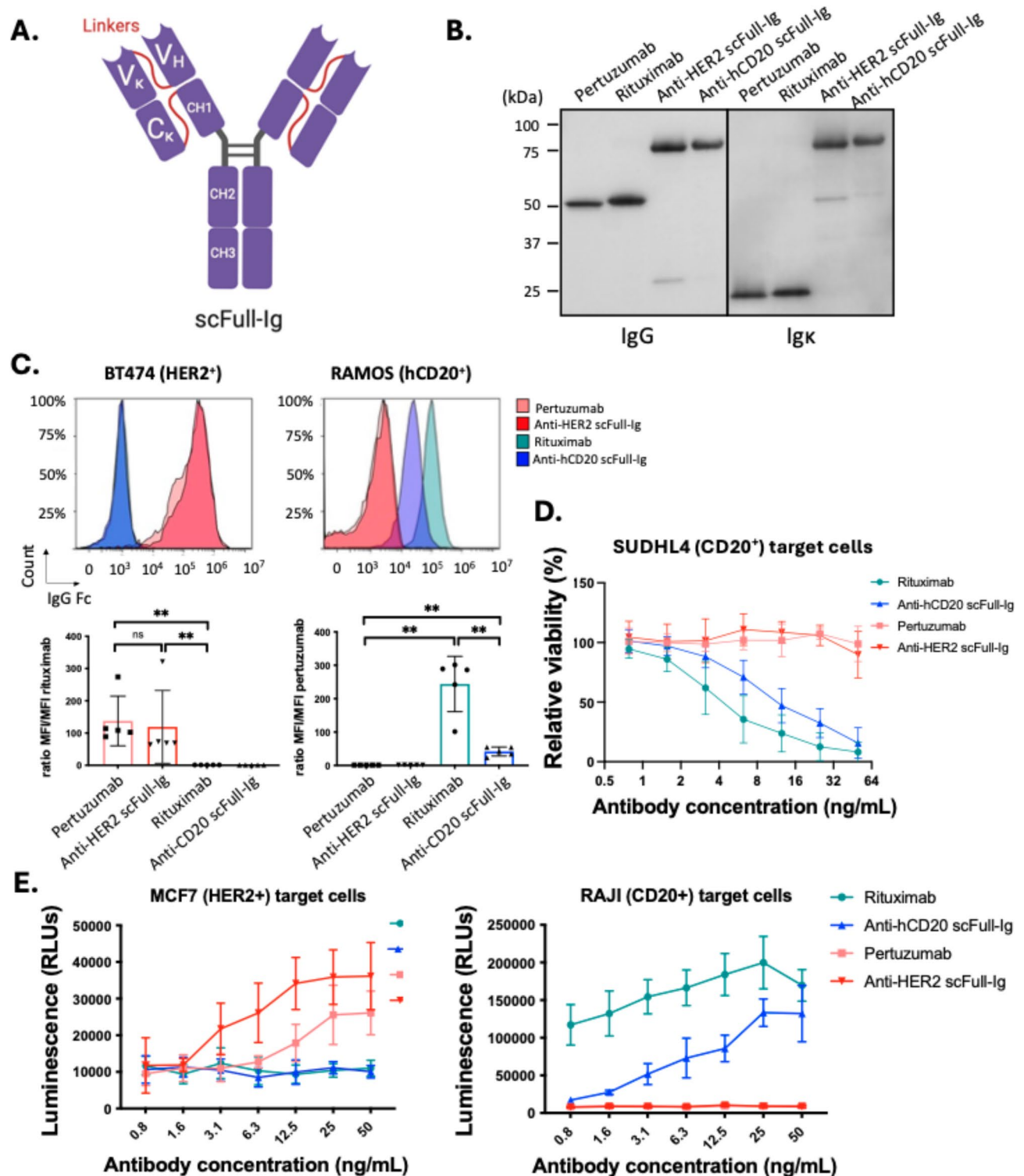


Fig. 1. Design and validation of scFull-Ig format. **A.** General architecture of the scFull-Ig design. **B.** Detection of scFull-Ig expression by western blot under reducing conditions. The membranes were stained with anti-IgG and anti-Igk antibodies. **C.** Validation of target binding by the soluble forms of anti-HER2 and anti-CD20 scFull-IgG. Below a representative cytogram, histograms show the ratio of the mean fluorescence intensity (MFI) for samples vs. negative controls (rituximab for HER2, pertuzumab for CD20); mean \pm s.d. ($n=5$), statistical significance with Mann-Whitney test is indicated (** $P<0.01$). **D.** Validation of the capacity of scFull-Ig to induce complement-dependent cytotoxicity. CD20⁺ SUDHL4 target cells and rabbit complement were used. **E.** Validation of the capacity of scFull-Ig to activate the ADCC-related reporter cell line Jurkat-Lucia NFAT-CD16 in the presence of cognate HER2⁺ MCF7 or hCD20⁺ RAJI target cells ($n=9$).

In addition to validation at the protein and DNA levels, in-frame RNA splicing of the inserted scFull-Ig USM cassette was checked by reverse transcribing and sequencing IgH μ transcripts from gene-edited cells, confirming the correct junction of the edited V-region cassette with the endogenous C μ exons (Fig. 2D).

While transcription of some upstream V genes can occur in B cell progenitors, KI of a pVH promoter and a V cassette downstream of JH6 is expected to hijack IgH locus expression in mature B cells, where VH gene transcription is restricted to the rearranged V gene that is the most proximal to the intronic transcriptional enhancer E μ and to the C region^{23,24}. That the editing of the IgH locus also disrupts endogenous IgH expression was verified by cell cytometry on two independent clones of DG75 edited cells, in which the homogeneous expression of the recombinant anti-HER2 scFull BCR was indeed accompanied by a complete loss of non-HER2 binding BCR molecules (Suppl. Figure 3 A).

To evaluate the functionality of the scFull-Ig USM BCR, edited DG75 B cells were stimulated in vitro with BCR ligands, permeabilized and monitored by cell cytometry for the phosphorylation of several relevant members of the BCR signaling cascade (Syk, BLNK, PLC γ , Erk and Btk) (Fig. 2E/Suppl. Figure 3B). BCR cross-linking by the multivalent form of HER2 Ag (biotinylated HER2 premixed with streptavidin) also triggered B-cell activation (Fig. 2E).

Thus, functional BCR expression through the KI of the scFull-Ig USM cassette is efficiently driven by CRISPR-mediated insertion into a B-cell line, prompting the extension of this strategy to primary B cells.

Preferential hypermutation of the scFv portion of the scFull-Ig USM in activated BL2 lymphoma cells

To evaluate the accessibility of the scFull-Ig USM to SHM, the anti-HER2 USM was inserted into the IgH locus of BL2, a human Burkitt lymphoma cell line prone to undergo SHM of its rearranged VDJ gene upon stimulation¹⁸. BL2 cells were then subjected to prolonged in vitro stimulation before final sequencing of IgH transcripts including the scFull module.

Within 41 variant sequences assembled and aligned to the expected 1133 bp long reference sequence (scFull USM spliced to C μ), 47 mutations were observed, 46 of which were located in the scFv region (25 in the VHDJH, most in the CDR3, 4 in the first linker, and 17 in the V κ J κ ; in parallel, only 1 of the 41 assembled sequences carried a mutation within the C κ sequence (Fig. 2F/Suppl. Figure 4).

Editing for membrane and secreted-form Ig expression and CSR in primary B cells

Primary B cells were magnetically sorted from buffy coats and cultured for two days in combination with the “1st step stimulation cocktail” (including CpG, anti-BCR, CD40L and IL-2). At day 2, CRISPR-mediated gene editing was performed under the same conditions as those used for DG75 cells but with the addition of the HDR enhancer (IDT) according to the supplier’s instructions. The expression of the anti-HER2 scFull-Ig USM antibody at the cell membrane was assessed at day 7. Approximately 6% of the cells were successfully edited and bound soluble HER2 when the most efficient donor DNA template was used (flanked with tCTS sequences) after gene editing in the presence of the HDR Enhancer (Fig. 3A). Like in DG75 cells, a multiplex competitive PCR assay was performed on DNA from edited primary B cells to validate the on-target integration of scFull-Ig USM (Fig. 3B).

Correct splicing of the HDR template to endogenous C exons was also confirmed by analyzing mature Ig transcripts, which subsequently evaluated the association of the scFull-Ig USM cassette with various IgH classes. While edited μ transcripts predominated at day 4, i.e., 2 days after transfection, their class-switched counterparts progressively increased during in vitro stimulation. With a significant increase from day 4 to day 10, in-frame splicing of the scFull-Ig USM KI cassette onto all endogenous Ig gene classes indicated the expression of not only IgM but also class-switched IgA, IgG and IgE (Fig. 3C).

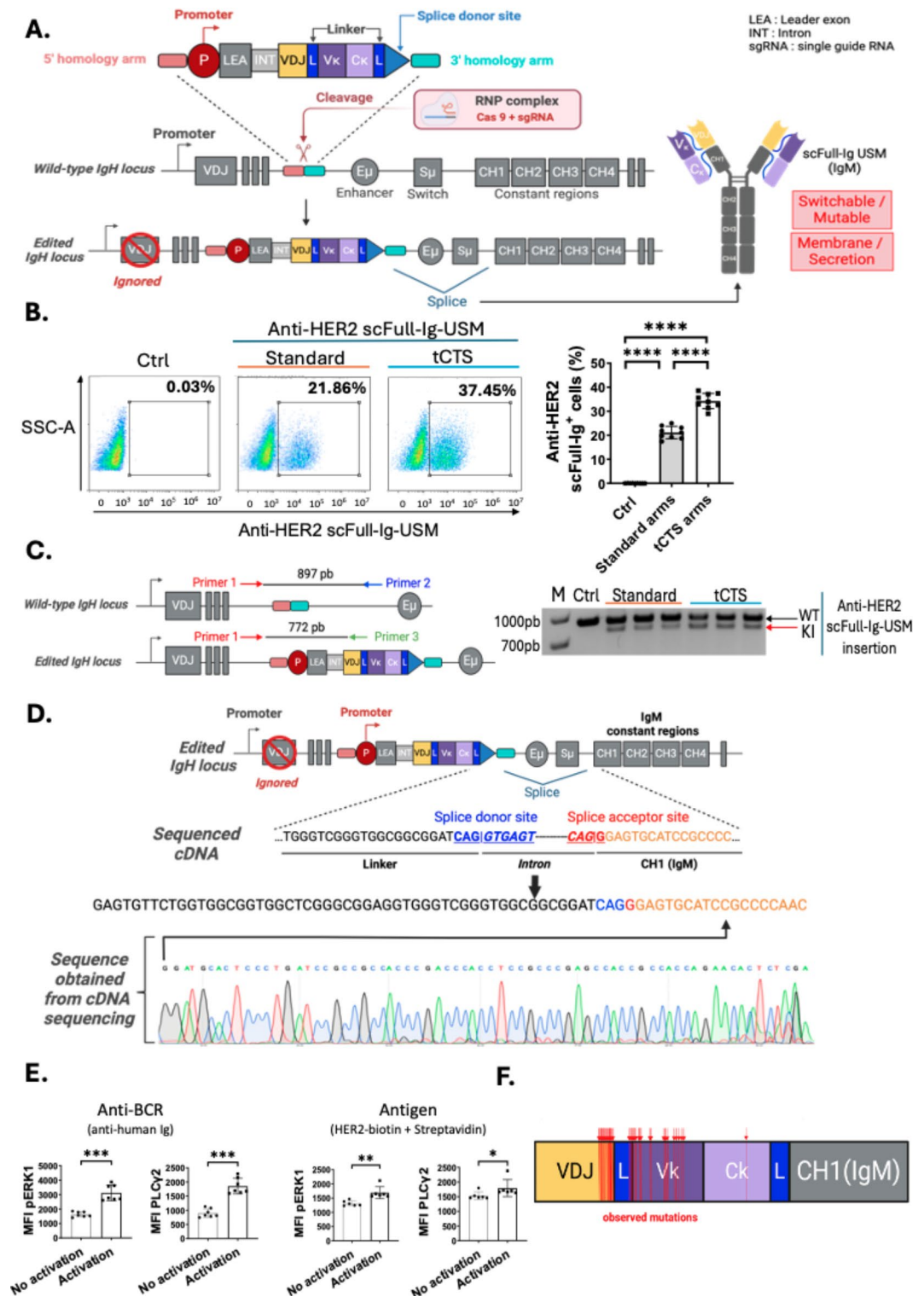
Accordingly, the secretion of HER-binding Abs in the supernatant of edited and in vitro-stimulated B cells was detected in parallel in day 7 and day 10 samples and included both IgM and class-switched Abs (IgG and IgA) (Fig. 3D/Suppl. Figure 5).

Overall, these data confirm that the KI of the [VDJ]_{peru}-Lnk-V κ J κ _{peru}-C κ -Lnk-J_H 3’splice cassette in the IgH locus upstream of the E μ enhancer allows the expression of scFull-Ig USM in primary B cells both in association with C μ and with downstream class-switched isotypes, and both as membrane-associated or secreted Ig.

Discussion

Adoptive cell therapy has developed remarkably over the last decade. While T cells are currently the mainstay of such strategies, B cells have recently attracted some interest. B-lineage cells can abundantly secrete proteins, and initial attempts to make them produce therapeutic proteins have relied on random lentiviral integration, particularly for the secretion of Ig or coagulation factors^{25,26}. However, for Ig, such strategies run the risk of generating cells with dual specificity and/or with a mixture of desired and undesired H-L associations. Precise CRISPR editing of Ig loci is thus attractive because it can simultaneously terminate the expression of endogenous Ig and replace it with a therapeutic mAb^{3–5}. In this context, the reformatting of the IgH locus is of particular interest due to its remarkable ability to yield either membrane-bound or secreted Ig and to regulate several other physiological variations in expression. With such intrinsic properties of the IgH locus, edited human B cells could fulfill two distinct and complementary therapeutic goals: either to secrete a mAb of interest on a long-term basis when differentiated into long-lived CD138⁺ CD20⁺ plasma cells or to patrol the patient’s lymphoid tissues as Ag-specific CD20⁺ CD27⁺ memory B cells to secrete the engineered Ig only upon encounter with Ag, and eventually further ameliorate their specificity.

We herein explored and validated a strategy for the modification of both Ig chain V domains through single editing of the IgH locus which retains its physiological organization, remains compatible with CSR and can then provide edited IgM molecules together with a variety of secondary class-switched variants carrying diversified



C regions, i.e., susceptible to exerting a diversified spectrum of effector functions. In addition, the proposed strategy joins the two edited V_H and V_L regions within a unique IgH locus upstream domain accessible to SHM. It is indeed known that the slope of the SHM rate in rearranged Ig V genes first increases until a maximum at about 0.5 kb from the functional transcription initiation site and then progressively decreases until reaching a basal level at about 1 to 1.5 kb from the transcription initiation site^{27,28}. An edited Ig could theoretically follow the path of affinity maturation and either increase its initial affinity for the target Ag or adapt to antigenic variations. The latter property would be of particular interest for the Ag variations found in chronic viral infections and in some cancer cells. Previous studies have proposed KI strategies for the IgH locus to express both the Ig H and L chains by covalently linking both chains through a long linker peptide or a cleavage site and then incorporating the IgL C sequence within the 0.5 to 1 kb interval most exposed to SHM downstream of the gene promoter⁴⁻⁷. In contrast, our strategy based on the expression of so-called scFull-Ig sequences starts with tandemly associated

◀ **Fig. 2.** Knock-in in the DG75 and BL2 cell lines. (A) Schematic of the insertion of a VDJ-Lnk-V κ -C κ -Lnk cassette between JH and the E μ enhancer, after CRISPR-mediated cleavage. “Created with BioRender.com.” (B) Expression of membrane-bound scFull-Ig USM: surface staining for anti-HER2 BCR was performed on gene-edited DG75 cells with biotinylated HER2 protein and PECE594-labeled streptavidin. Mean \pm s.d. ($n=9$). Statistical significance with Mann-Whitney test is indicated as follows: **** $P < 0.0001$. (C) Validation of the KI at the DNA level by multiplex competitive PCR. (D) Validation of correct splicing of the USM on the endogenous C μ CH1 exon. RNA was extracted from the edited cells, splicing of the USM to C μ was confirmed by amplification and sequencing of the specific band. (E) Detection by flow cytometry of the intracellular phosphorylation of members of the BCR signaling cascade (PLC γ 2 and pERK1) after cross-linking of the membrane-anchored anti-HER2 scFull BCR ($n=7$) either with anti-human Ig or with precomplexed fluorescent Ag [biotinylated HER2 + streptavidin-PECE594] ($n=6$). Significance according to Mann-Whitney test is indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $P < 0.0001$). (F) Schematic of the positions of mutations observed in the scFull USM KI in the IgH locus of BL2 cells, after *in vitro* stimulation (arrows indicate positions of individual mutations).

V $_H$ and V $_L$ sequences in an upstream position which thereby hijack IgH production in B cells. Although based on a single edit of the IgH locus, the scFull-Ig USM design therefore combines several advantages. This approach simplifies gene editing, guarantees the assembly of given IgH and IgL chains into a unique full Ig molecule, and complies with the goal of including both the V $_H$ and V $_L$ regions in an upstream transcribed IgH domain exposed to high SHM in activated B lymphocytes, while keeping the C κ region less exposed to SHM. Another important expected outcome of such an editing of the IgH locus is the expression of both membrane-associated BCR and secreted antibodies with complete and switchable endogenous Fc domains, thereby capturing all Ig effector functions. The objectives of this study were to validate the functionality of the scFull-Ig USM format in terms of efficient membrane expression, secretion, Ag specificity, and ability of the scFv domain to functionally associate with the various endogenous Fc domains in chimeric molecules after an appropriate IgH KI.

Linkers incorporated into Fv domains may eventually affect the conformation of Ig, and preservation of Ag specificity and/or affinity is a preliminary requirement for a mAb expressed in a single-chain format²¹. Accordingly, we verified the retained Ag binding of two classical therapeutic mAbs, anti-CD20 rituximab and anti-HER2 pertuzumab, when modified according to the scFull design. In both cases, antigen specificity was retained but somewhat lower binding was observed with rituximab in the scFull format, in agreement with the findings of a previous study with other single-chain derivatives of rituximab²¹. In addition to binding, we also verified that secreted scFull-IgG, with a fully preserved Fc region, retains the ability to efficiently induce ADCC and CDC against target cells.

Using CRISPR-based editing of the IgH locus and KI of scFull-Ig USM cassettes, we confirmed in the HER2 model that both human B-cell lines and human primary B cells can be efficiently edited for Ig expression: the upstream part of the cassette, including both V regions and the C $_L$ exon, was correctly spliced onto the endogenous IgH C regions, and edited B lymphocytes hereby expressed a membrane BCR with the expected Ag specificity. Like its wild-type counterpart, this edited BCR was connected to the intracellular signaling cascade and induced intracellular phosphorylation upon BCR cross-linking by specific antibodies or by a specific Ag. Transcript sequencing also revealed that scFull-Ig USM expressed in primary B cells was initially associated predominantly with IgM but later also associated with class-switched IgG, IgA and IgE. The observation that the number of class-switched scFull transcripts increased with time in *in vitro*-stimulated B cells showed that they mostly originated from ongoing CSR events occurring in culture after IgH locus CRISPR editing. This is notably true for the ϵ transcripts since IgE⁺ cells are short-lived *in vivo* and absent from normal PBMCs²⁹.

Many adjustments need to be made before edited human B cells become good candidates for cell therapy, but improving the efficacy of KI in these cells is an important challenge. First, this relies on the method of delivery of the DNA template to B cells, which must combine efficiency with low toxicity. We decided to focus our efforts on a virus-free protocol for which safe and well-designed production and purification methods are easily transferable under good manufacturing practice (GMP) conditions, as required for future preclinical development. In terms of safety, naked DNA-based strategies can compete with other virus-free methods based on nanoparticles and mRNAs^{30–32} but can additionally provide stable expression.

One limitation of B-cell editing is the difficulty of performing HDR with an exogenous DNA template at the optimal insertion site of the IgH locus. We expressed gene cassettes after their KI at the IgH locus using an efficient protocol for HDR-mediated precise insertion in viable B cells, with an optimized Cas9/sgRNA ratio, amount of DNA template, addition of HDR-enhancing molecules and optimized primary B-cell culture conditions prior to and after the KI experiment. The rate and homogeneity of the KI were subsequently improved by using the tCTS-modified dsDNA template, as described by Nguyen et al.²². Overall, although the protocol proposed in this study is efficient, further optimization will remain useful. In the future, strategies that block the nonhomologous end-joining (NHEJ) pathway³³ may also help promote HDR. As B cells express high levels of intracellular Toll-like receptors, the HDR rate and cell survival could be further improved by using ssDNA templates³⁴ or by inhibiting DNA sensors to reduce the toxicity of transfected DNA, as reported for T-cell editing³⁵.

In conclusion, we report an efficient strategy for editing human B cells by a single precise genetic modification of the IgH locus. This disrupts the expression of the endogenous V $_H$ domain and replaces it with a cassette providing most of the [Fv/C $_L$] region of a mAb of interest, albeit in a single-chain format. This strategy rebuilds a complete Ig by reassociating the KI [Fv/C $_L$] region with the endogenous [CH1 + Fc] region encoded by the IgH locus, thereby guaranteeing the expression of the IgH chain under all possible normal variations, either expressed at the cell membrane as a BCR in B lymphocytes or eventually switched or mutated once these cells

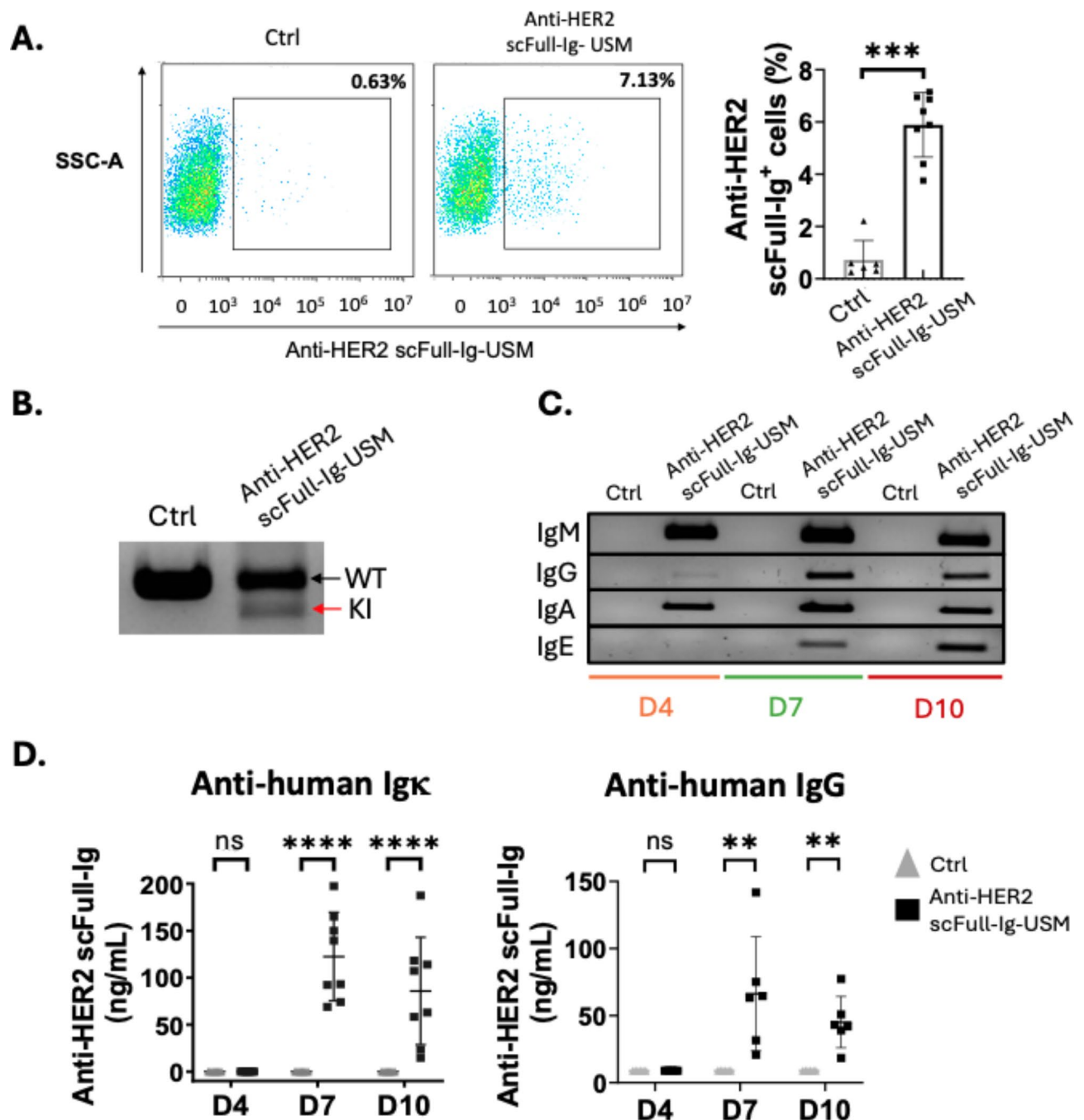


Fig. 3. Knock-in in primary B cells. **(A)** Expression of the membrane-bound scFull-Ig USM: cell surface staining for the anti-HER2 BCR was performed as in Fig. 2. Mean \pm s.d., $n = 9$. Statistical significance with Mann-Whitney test is indicated as follows: *** $P < 0.001$. **(B)** DNA was extracted from edited primary B cells, and PCR was performed with 3 primers to confirm the correct integration of the template ($n = 4$). **(C)** RNA was extracted from edited primary B cells, and splicing was confirmed by performing RT-PCR on IgH transcripts from the unswitched C μ gene or C γ , C α and C ϵ genes after CSR ($n = 6$). **(D)** Detection of secreted anti-HER2 scFull-Ig USM by ELISA: supernatants of gene-edited cells were collected at day 4 (D4), 7 (D7) and 10 (D10). Total and IgG-class HER2-specific Abs were evaluated by ELISA using anti-k or anti g chain secondary antisera, respectively, compared to standard dilutions of commercial pertuzumab ($n = 8$ or $n = 6$).

have been activated, or finally secreted by differentiated plasma cells. Edited B cells will then be ready for transfer into recipient mouse models to assess antibody secretion and potential adoptive humoral immunity.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization, M.Co.; Methodology, M.Co., N.A, M.Ca, S.D., J.L., L.D & Y.D.; Formal Analysis, M.Co., N.A, M.Ca , C.S., J.M., A.G. & Y.D; Writing – Original Draft, M.Co., N.A, M.Ca & Y.D.; Writing – Review & Editing, M.Co; Funding Acquisition, M.Co & A.G.; Supervision, M.Co. & Y.D.All authors reviewed the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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