TITLE

A highly compact epitope-based marker / suicide gene for easier and safer T-cell therapy.

SHORT TITLE

RQR8 as a universal safety switch

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KEY POINTS

Marker-genes enable detection and selection of T-cells, while suicide-genes enable selective destruction of T-cells in case of toxicity

RQR8 is a 136 amino acid epitope-based marker/suicide gene that enables clinical selection, cell tracking and deletion in case of toxicity

ABSTRACT

A compact marker / suicide gene which utilizes established clinical grade reagents and pharmaceuticals would be of considerable practical utility to T-cell cancer gene therapy. Marker genes enable measurement of transduction and allow selection of transduced cells, while suicide genes allow selective deletion of administered T-cells in the face of toxicity. We have created a highly compact marker/suicide gene for T-cells combining target epitopes from both CD34 and CD20 antigens (RQR8). This construct allows selection with the clinically approved CliniMACS CD34 system (Miltenyi). Further, the construct binds the widely used pharmaceutical antibody Rituximab, resulting in selective deletion of transgene expressing cells. We have tested functionality of RQR8 in vitro and in vivo, as well as in combination with T-cell engineering components. We predict that RQR8 will make T-cell gene-therapy both safer and cheaper.

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INTRODUCTION

Adoptive immunotherapy is an established and evolving therapeutic approach. In the setting of allogeneic haematopoietic stem cell transplantation (HSCT), donor lymphocyte infusions (DLI) are frequently given to treat relapse of haematological malignancies. Tumour infiltrating lymphocytes (TILs) are effective in treating metastatic melanoma. Genetic engineering of T-cells greatly increases the scope and potency of T-cell therapy: T-cell receptor transfer allows targeting of intracellular cancer antigens, while chimeric antigen receptors (CAR) allow targeting of surface cancer or lineage specific antigens[1]. Clinical responses have been observed with both approaches, and numerous further trials are underway.

Acute adverse events can occur following adoptive immunotherapy. Graft-versus-host disease (GvHD) is a common and serious complication of DLI. Administration of engineered T-cells has also resulted in toxicity[2-5]. For instance, on-target off-tumour toxicity has been reported in native T-cell receptor transfer studies against melanoma antigens[6]; T-cells re-directed to the renal cell carcinoma antigen carbonic anhydrase IX (CAIX) produced unexpected hepatotoxicity[7]. Immune activation syndromes have been reported after CD19 CAR therapy[3,8,9]. Finally vector-induced insertional mutagenesis results in a theoretical risk of lymphoproliferative disorders. The incidence and severity of these toxicities is unpredictable. Further, in contrast to a therapeutic protein or small molecules whose adverse events usually abate with the half-life of the therapeutic, T-cells engraft and replicate, potentially resulting in escalating and fulminant toxicity.

A suicide-gene is a genetically encoded mechanism which allows selective destruction of adoptively transferred T-cells in the face of unacceptable toxicity. Two suicide-genes have been tested in clinical studies: Herpes Simplex Virus thymidine kinase (HSV-TK)[10] and inducible caspase 9 (iCasp9)[11]. Expression of HSV-TK in T-cells confers susceptibility to ganciclovir. HSV-TK is a highly effective suicide-gene but immunogenicity limits application to clinical settings of profound immunosuppression such as haploidentical HSCT. Further, it precludes the use of Ganciclovir for treatment of cytomegalovirus (CMV) infection. iCasp9 is activated by an experimental small molecule dimerizer (AP20187), hence use of this suicide-gene depends on availability of clinical-grade dimerizer. Finally, both are intracellular proteins and typically must be co-expressed with a marker-gene.

Marker-genes enable detection and positive selection of transduced T-cells. Some T-cell engineering strategies do not result in transgenic expression of readily detectable surface proteins[1]. In these cases, measurement of transduction and tracking of cells in peripheral blood is difficult, and a marker-gene is useful. Further, in some settings, it is essential to administer only transduced T-cells, for instance in GvHD suicide-gene protocols. Described marker-genes include several truncated type I transmembrane proteins not normally expressed on T-cells: the truncated low-affinity Nerve Growth Factor (dLNGFR)[12], truncated CD19[11] and truncated CD34[13]. A particularly attractive feature of CD19 and CD34 is the availability of off-the-shelf Miltenyi CliniMACS selection system for clinical grade sorting. However, these are relatively large surface proteins which may tax the vector packaging capacity and transcriptional efficiency of an integrating vector.

We sought to develop a highly compact epitope-based construct that would act as both suicide and marker gene. For maximum convenience, we restricted our design to one which could utilize existing GMP reagents and standard pharmaceuticals for both selection and deletion. Our final construct RQR8 is a 136 amino acid protein which is recognized by the anti-CD34 antibody QBEnd10, the antibody used in Miltenyi CliniMACS CD34 selection system. It renders T-cells highly susceptible to lysis by the therapeutic monoclonal Rituximab. We anticipate RQR8 will make T-cell therapy cheaper, easier and safer.

MATERIALS AND METHODS

Cloning: All constructs were generated by in-house gene-synthesis using PCR assembly of overlapping oligos unless otherwise specified. Codon-optimization used an in-house algorithm (written by MP, available on request): strove to keep GC content at 70%, eliminate cryptic splicing, harpins, literal repeats and any possible cis-acting sequences. Identity of constructs was confirmed through capillary sequencing Applied Biosystems 3730xL capillary. Phusion polymerase, quick Ligase and NEB5α (New England Biolabs) were used for molecular cloning. Oligonucleotides were purchased from IDTDNA. The retroviral vector used in all constructs was the splicing oncoretroviral vector SFG[14]. Enhanced green fluorescent protein (eGFP) and enhanced blue fluorescent protein 2 (eBFP2)[15] were co-expressed from an ECMV IRES sequence[16]. Anti-GD2 CAR was as described <paper in press>. RQR8 was co-expressed with CAR by cloning it upstream of the CAR separated by an in-frame foot-and-mouth-like 2A peptide TaV[17], or co-expressed with the WT-1 specific TCRα and β chains with two interposed FMD-2A TaV sequences, codon wobbled to prevent retroviral recombination. Annotated sequences of constructs included in supplementary data.

Retroviral production and transduction: RD114 pseudotyped supernatant was generated as follows: 293T cells were transfected with vector plasmid; RDF, an expression plasmid to supply RD114 envelope (gift of Mary Collins, University College London)[18] and PeqPam-env, a gagpol expression plasmid (gift of Elio Vanin, Baylor College of Medicine)[17,19,20]. Transfection facilitated using genejuice (Merck). Eco pseudotyped supernatant was generated in a similar way with RDF substituted by the ecotropic envelope expression plasmid pMono.Eco. PBMC transductions were performed as follows: T-cells were isolated by Ficoll (GE Healthcare) gradient centrifugation and stimulated with PHA at 5µg/ml. IL2 stimulation (100i.u./ml) was added following overnight stimulation. On day 3, T-cells were harvested, plated on retronectin and retroviral supernatant and centrifuged at 1000xG for 40 minutes. Murine splenocytes were isolated and transduced as follows: Splenocytes from 6-8 week old C57BL/6 mice were isolated by passage through a 40µm pore sieve followed by RBC lysis in ACK lysis buffer (Lonza). Splenocytes were activated in RPMI supplemented with 10% FCS, glutamax, 10mM HEPES, 0.1µm 2-mercaptoethanol) supplemented with 2µg/ml Concanavalin A (Sigma), 1ng/ml IL7 (Peprotech) at a density of 1 to 1.5 x 10^6/ml for 24 hours. Retroviral transduction was performed by loading retronectin coated plates (Takara) with 2 to 3 x 10^6 splenocytes suspended in 1ml retroviral supernatant and centrifuged at 805G for 90 minutes then recovered by overnight incubation at 37ºC. The following day, splenocytes were recovered from the plate, resuspended in complete RPMI supplemented with 50ng/ml murine IL2 (Invitrogen) and cultured overnight. Transduced splenocytes were purified on LS columns using CD34 microbeads (Miltenyi Biotec) as per manufacturer's instructions. Following positive selection, splenocytes were resuspended in conditioned media retained from the overnight IL2 stimulation and cultured overnight. Purity and viability of selected splenocytes were assessed by flow cytometry prior to adoptive transfer 1 day following positive selection.

Antibodies, flow-cytometry and flow-sorting: Chimeric antigen receptor was stained using a Cy5-conjugated polyclonal goat anti-human-Fc (Jackson Immunotech). Rituximab and Ofatumumab were procured from the hospital pharmacy at University College London Hospital. Murine IgG2a rituximab was generated by cloning the variable regions of the heavy and light chain from IDEC2B8 hybridoma HB-11388 checked against patent 5,843,439 into the constant regions of murine IgG2a and murine Immunoglobulin kappa (sourced from Invivogen plasmids pFUSEss-CH1g-mG2A and pFUSE2ss-CLIg-mk) into a retroviral vector (MP9201 SFG.S-Rtx_H_mIgG2a.I2.eGFP and MP9202 SFG.S-Rtx_L_mKappa.I2.eBFP2; see supplementary data for sequences). K562 cells were transduced with vectors coding for both heavy and light chains. A highly productive clone was identified, expanded in phenol red- IMDM supplemented with 2.5% IgG depleted serum (Biosera). Flow-cytometry was performed using Beckman-Coulter Cyan and BD LSRII Fortessa instruments. Flow cytometric sorting was performed using a Beckman Coulter MoFlo-XDP sorter.

Complement mediated Cytotoxicity. Transduced T-cells were exposed to 25% baby-rabbit complement (AbD Serotec) for 4 hours with or without inclusion of Rituximab (100µg/ml) to examine CDC-mediated sensitivity. Miltenyi CD34 magnetic bead selected transduced RQR8 T-cells were compared against a similarly treated population of Q8 transduced T-cells to demonstrate specificity of CDC-mediated deletion. Further examination of CDC assay parameters was achieved through time-course/dose titration assays using RQR8 transduced T-cells incubated with Rituximab at 12.5, 25, 50 and 100µg/ml, and timepoint assessments ranging between 1 to 120 minutes.

Antibody dependent Cellular Cytotoxicity. NK cell effectors were generated using a K562 stimulator cell-line (after Campana[21]), expressing membrane-bound IL15 and 41BBL established by retroviral vector transduction and single-cell cloning. Freshly isolated peripheral blood mononuclear cells from healthy donors were co-cultured 1:1 in 24-well TC-treated multiwell plates with irradiated K562.41BBL.mIL15 irradiated at 120Gy and supplemented with 40iu IL2. Partial media changes performed as required. Following 7 days in culture, a pure population >95% purity of NK cells were isolated following a single round of Miltenyi CD56 positive selection. Transduced T-cell targets, were co-cultured with NK cell effectors at effector:target ratios of 16:1, 8:1, 4:1 and 2:1 for 48 hours. Cellular deletion was assessed by flow cytometry following Annexin V (BD Biosciences) and Propidium Iodide (Sigma) staining. For sensitivity assessment of ADCC mediated deletion, NK cell effectors were prepared with CellTRACE violet (Invitrogen) immediately prior to assay set-up. For ADCC specificity assessment, NK cell effectors remained unstained, with targets composed of a Miltenyi CD34 magnetic bead-selected mixture of Q8 and RQR8 transduced T-cells in equal proportion with Q8 and RQR8 targets identified by expression of eBFP2 or eGFP fluorescent proteins respectively.

Chromium Release Cytotoxicity Assay. Cytotoxicity of CAR T-cells was evaluated in a standard 4-hour ^{51}Cr release assay as previously described[22].

***In vivo* engraftment model.** This work was performed under UK home-office approved project license and in accordance with institutional policies. Splenocytes from C57BL/6 mice were transduced with SFGmR.RQR8 in the absence of stimulation following 24 hours of stimulation by Con A/IL7 (2µg/ml and 1ng/ml respectively). Following transduction, splenocytes were re-stimulated with IL2 (50ng/ml) and cultured overnight. Following a single round of Miltenyi CD34 selection, purity >90% was achieved. $1.5x10^6$ transgenic splenocytes were administered by tail vein injection to (C57BL/6 x Balb/c) F1 recipients 4 hours following 5Gy X-ray irradiation pre-conditioning. 7 days post-transfer, engraftment was assessed by QBEnd10 staining of peripheral blood. 150µg Ritux-mIgG2a was administered by tail-vein injection on days 7, 10 and 12 post-transfer. Mice were sacrificed on day 14 with samples taken from peripheral blood, spleen, lymph nodes and bone marrow stained for CD4, CD8, $H2K^d$ and QBEnd10 to assess engraftment.

RESULTS

The monoclonal antibody QBEnd10 binds a linear 16 residue fragment of CD34. We first identified the binding site of the type I anti-CD34 mAb QBEnd10. A DNA library coding for overlapping 31 residue fragments of the serine/threonine rich amino-terminus of CD34 was generated. This library was cloned into a retroviral vector between CD34 signal peptide and trans-membrane domain. SupT1 cells were transduced with the subsequent retroviral library. QBEnd10 binding cells were single-cell sorted and the retroviral integrant subsequently sequenced from genomic DNA (not shown). From this, we determined that QBEnd10 binds to the amino-terminal 40 amino acids of mature CD34. We hypothesized that this epitope might need to be projected from the cell surface and allowed loose orientation to allow effective QBEnd10 binding. The CD8 stalk is comprised of only 42 amino acids, but results in a projection sufficient to allow the globular CD8 amino terminus to reach past the TCR and interact with MHC class I on a neighbouring cell[23]. By transducing SupT1 cells with equal titre of retroviral supernatant, we then compared QBEnd10 binding to full-length CD34 with the above 40 residue epitope connected directly to the CD8 trans-membrane domain, fused directly to the CD8 stalk, or connected to the CD8 stalk via a serine-glycine linker. Both constructs with the CD8 stalk exhibited similar binding to full-length CD34 suggesting that the CD8 stalk enhances access of QBEnd10 to the epitope. [Figure 1(a) and 1(b)]. Finally, we refined the epitope further by sequential deletion of the amino- and carboxy-termini. We concluded that a 16 residue linear sequence of CD34 on a CD8 stalk could recapitulate equivalent binding of QBEnd10 as the full CD34 protein. [Figure 1(c)].

Rituximab binding equivalent to that of full-length CD20 could only be achieved with a mimotope. We next sought to achieve binding by Rituximab to a fragment of CD20 in an analogous fashion: The binding site of Rituximab to CD20 has been elucidated by deletion studies[24] and crystallography[25]: Rituximab binds to the disulphide-constrained portion of the CD20 major extracellular loop. We generated constructs where this disulphide constrained loop was expressed on the CD8 stalk with no flanking residues (dCD20-loop v1) or 4 additional residues of CD20 on either side (dCD20-loop v2). Construct dCD20-loop v2 did not bind Rituximab, while dCD20-loop v1 achieved only very poor binding. [Figure 1(d)]. Perosa et al[26] described Rituximab binding mimotopes of CD20. These sequences resemble the major extracellular loop of CD20 and compete with Rituximab for CD20 binding. Hence, we next generated constructs connecting these mimotopes to the CD8 stalk: connecting a circular mimotope to the CD8 stalk. This could re-capitulate binding of Rituximab. We next generated constructs with the QBEnd10 epitope linked to the CD20 mimotope in either orientation (constructs termed RQ8 and QR8 respectively). [Figure 2(a)]. This circular mimotope and the QBEnd10 epitope described above could be co-expressed in either orientation on CD8 stalk with no diminution of binding of either antibody. [Figure 2(b)].

Two copies of Rituximab mimotope flanking the QBEnd10 epitope on the CD8 is optimal for Rituximab mediated lysis. Despite binding of Rituximab, CDC assays with RQ8 and QR8 showed significantly less lysis than with full-length CD20. We next proceeded to try to gain some insight into why this was and to improve killing. The circular rituximab mimotope was placed into several formats: RCH2CH3 was constructed by cloning the mimotope onto a human IgG1 Fc spacer. RRQ8 was constructed by linking two copies of the rituximab mimotope followed by the QBEnd10 epitope and CD8 stalk. RQR8 has two copies of the Rituximab mimotope flanking the QBEnd10 epitope on the CD8 stalk. [Illustrated Figure 2(a)]. Primary human T-cells were transduced with these constructs, along with Q8 and full-length codon-optimized CD20. CDC and ADCC assays were performed on unsorted T-cell populations. Constructs RQ8 and RQR8 resulted in ADCC equal to that of CD20, while only RQR8 resulted in CDC equal to that of the full-length protein ($p<0.05$). We hence selected RQR8 for further study.

RQR8 has properties which make it a good marker-suicide gene. We first explored utility of RQR8 as a marker-gene. Primary human T-cells transduced with RQR8 could be sorted to high stringency with

Miltenyi CD34 beads. [Figure 3(a)]. Further, as few as 0.01% RQR8 transduced primary human T-cells could be detected in serial dilution experiments. [Supplementary Figure 1]. Further, RQR8 allows immunohistochemical detection with QBEnd10. Concomitant staining for CD3 or with another CD34 antibody will easily discriminate transgenic T-cells from other CD34 cells such as HSC or endothelium. [Supplementary Figure 2]. Next, we explored utility of RQR8 as a suicide gene: Miltenyi QBEnd10 bead sorted T-cells are very efficiently lysed by a single exposure to Rituximab and complement: In 50:50 mixing assays, were sorted T-cells were mixed back with non-transduced T-cells, 98% of transduced T-cells could be depleted by Rituximab and complement. [Figure 3(b)]. Both CDC and ADCC mediated sensitivities of RQR8 are equal to that demonstrated by expression of full length codon-optimized CD20 [Figure 2(b) and Figure 2(c)]. Rituximab-mediated deletion is efficient at 25μg/ml which is well within the therapeutic window of this agent[27]. [Figure 3(c)] CDC mediated killing of RQR8 expressing T-cells occurs rapidly with maximum lysis within 30 minutes of exposure. [Figure 3(c)].

RQR8 is of utility when expressed with chimeric antigen receptors. Chimeric antigen receptors (CARs) are amongst the most commonly used T-cell engineering components. We co-expressed RQR8 with an anti-GD2 CAR in a retroviral vector using the self-cleaving foot-and-mouth disease 2A sequence[17]. This resulted in obligate co-expression of CAR and RQR8 [Figure 4(b)]. Selection with QBEnd10 beads resulted in a pure population of CAR expressing T-cells (>95% from 6 separate experiments). We next mixed RQR8 sorted T-cells 1:1 with non-transduced T-cells and performed lysis and functional experiments. CDC by Rituximab led to deletion of >97% of the CAR expressing population. [Figure 4(c)] Further, this depleted population completely lost recognition of antigen expressing targets. [Figure 4(d)]. RQR8 could also be co-expressed in a tricistronic vector with a native alpha/beta T-cell receptor with FMD-2A like sequences (not shown).

T-cells expressing RQR8 are not susceptible to lysis by Ofatumumab. Recent clinical studies of adoptive immunotherapy with CAR engineered T-cells have focused on B-cell malignancies. In this setting, concomitant administration of a B-cell depleting therapeutic antibody might be desirable. Given the long in vivo half-life of Rituximab, its inclusion in a conditioning regimen precludes use of RQR8 in the vector. A new generation of anti-CD20 therapeutic antibodies has however been developed. One such agent, Ofatumumab, binds to a different epitope on CD20 than that of Rituximab[28]. We investigated whether Ofatumumab would bind to and cause lysis of RQR8 expressing cells. First, T-cells transduced with Q8, CD20 and RQR8 were stained with Rituximab and Ofatumumab [Figure 5(a)]. While CD20 expressing T-cells bound both antibodies, RQR8 expressing T-cells only bound Rituximab. Further, a CDC assay showed lysis of CD20 expressing T-cells with both Ofatumumab and Rituximab [Figure 5(b)], while only Rituximab was capable of lysing RQR8-expressing T-cells. Hence Ofatumumab can be used in a preparative regimen of engineered T-cell therapy with RQR8. Binding of a panel of other anti-CD20 mAbs in clinical use or pre-clinical development to RQR8 is detailed in supplementary table 1.

T-cells transduced with RQR8 can be efficiently deleted in vivo. We used an immunocompetent haploidentical adoptive transfer model with RQR8 transduced C57BL/6 splenocytes transferred to non-lethally irradiated C57BL/6 x Balb/c cross (F1) recipients. This model allows good engraftment levels in all lymphoid tissue sustained by allogeneic stimulation, but also preserves endogenous lymphocytes. Rituximab is a chimeric antibody with mouse variable regions and human kappa/IgG1 constant regions with limited activity in mice. So to more truly determine potency of deletion, we re-engineered Rituximab to mouse IgG2a, the functional equivalent of human IgG1[29], supplementary methods and supplementary figure **3** (referred henceforth as ritux-mIgG2a). $1.5x10^6$ RQR8 transduced QBEnd10 sorted splenocytes resulted in engraftment readily detectable at day 7 in all mice. After one week of ritux-mIgG2a administration, transgenic T-cells were no-longer detectable in peripheral blood by flow cytometry ($p<0.001$), while marking levels continued to increase in the control cohort [Figure 6(a)]. Mice were then sacrificed and assessed for engraftment in lymph nodes, bone-marrow and spleen. Depletion levels of 96%, 98% and 99% were observed ($p<0.001$) in blood, bone-marrow, lymph-nodes and spleen [Figure 6(b) and 6(c)]. This depletion was in face of the considerable engraftment evident in bone-marrow, mean

35% and spleen mean 22%. [Figure 6(c)]. RQR8 allows for rapid and highly effective in vivo depletion of transgenic T-cells in all lymphoid compartments. In addition, we performed an *in vivo* time-course with separate cohorts of mice sacrificed at different time-points. Supplementary figure 4 shows depletion at 6, 48 and 168 hours in peripheral blood within individual mice. Supplementary figure 5 shows depletion in blood, spleen, bone marrow and lymph nodes in different cohorts of mice at 6, 24, 48, 120 and 168 hour time-points. This demonstrates over 50, 60 and 70% depletion in spleen, bone-marrow and blood respectively within 6 hours post rituximab therapy. Dynamics of increasing engraftment in control animals and increasingly effective depletion in Ritux-IgG2a recipients is observed.

DISCUSSION

Genetic engineering of T-cells is increasing the scope and application of adoptive immunotherapy. Unlike traditional small molecule or protein therapeutics, adoptive immunotherapy aims to achieve engraftment of autonomous cells with the capacity to persist indefinitely and proliferate. Hence, toxicity may be long-lived and may escalate. Further, cellular therapies are highly complex and optimization of several parameters such as transgene and cellular production with rapid iterative clinical experiments are likely needed before development of phase II/III studies. A simple compact T-cell engineering component that facilitates production and allows selective depletion in clinical study subjects without requirement for new clinical-grade reagents would be of value to the field.

Existing strategies which have been clinically tested to allow selective depletion of transgenic T-cells (suicide-genes) include the Herpes Virus Thymidine Kinase (HSV-TK) gene and inducible Caspase 9 (iCasp9). The former renders T-cells susceptible to Ganciclovir, while the latter[22] is activated by a small molecular chemical inducer of dimerization (CID). HSV-TK is an effective suicide-gene[10] but, pre-formed immune responses may restrict its use to clinical settings where there is marked immunosuppression such as in haploidentical stem cell transplant recipients[30,31] iCasp9 is also effective but its use is dependent on the availability of a proprietary CID manufactured to clinical grade.

Both HSV-TK and iCasp9 are intracellular proteins which prevents their use as simultaneous marker-genes which would allow tracking engraftment and the selection and purification of transduced cells prior to administration. Truncated CD34 and truncated CD19 have been co-expressed with HSV-TK and iCasp9 respectively as marker-genes; both allow clinical-grade Miltenyi cliniMACS sorting of transduced T-cells, as well as tracking of transduced cells by flow cytometry in samples from study subjects. However, CD19 is currently the prime target for CAR therapy and using this antigen as a marker precludes concomitant targeting. Truncated or full-length CD34 may impact on the migratory ability of modified cells[32]. Further, a combined marker and suicide-gene adds a considerable genetic payload to the gene vector.

Therefore we reasoned that we could construct a highly compact combined sort-suicide gene using antibody binding epitopes from both CD34 and CD20. Further, by aiming to recapitulate the properties of CD34 as a marker-gene and CD20 as a suicide-gene, our construct could be used with "off-the-shelf" reagents. First we determined that the epitope of CD34 recognized by QBEnd10 was a linear peptide localised to the extreme amino-terminus of the protein. The crystallographic data of the CD20 molecule suggested that the major extracellular loop was all that was required for binding to Rituximab and we were surprised when this loop failed to afford full binding. We concluded that correct orientation of this loop only occurs in full-length CD20 and replacing this loop with a mimotope afforded a solution. The need for two copies of the Rituximab binding mimotope to result in effective CDC may be explained by the requirement to cluster CD20 to effect lysis. Our final homodimeric (and hence tetravalent) RQR8 allows cross-linking and clustering which may resemble that seen by the native antigen CD20.

With all non-native genes used to engineer T-cells, immunogenicity presents a potential limitation. For instance, chimeric antigen receptors are typically derived from murine antibodies and have many junctional and linker sequences exposed. Similarly, RQR8 has junctional sequences which may be immunogenic. A bioinformatics analysis of B- and T-cell immunogenicity of RQR8 is presented in supplementary figure 6, identifying 6 T-cell and 24 B-cell epitopes. It should be noted that current CAR and TCR adoptive immunotherapy protocols typically employ profoundly lymphodepletion preparatory

regimens resulting in profound immunosuppression at the time of T-cell administration. This may reduce the occurrence of, and may prevent development of an immune response. Ultimately, only clinical studies can determine the practical consequences of immunogenicity of any particular T-cell engineering component.

Suicide-gene strategies based on transgenic expression of a binding target for a therapeutic antibody have been proposed[33-36] with a Cetuximab based system[34] being the closest to RQR8. Here, truncated EGFR is expressed on a T-cell and depletion effected by the anti-EGFR therapeutic mAb Cetuximab. This system is less likely to be immunogenic than RQR8. However, RQR8 has some advantages: Firstly, RQR8 has an "off-the-shelf" clinical grade selection system available; Secondly Rituximab monotherapy is well tolerated by the majority of patients with little increase in opportunistic infection[37,38] and no maximally tolerated dose. In contrast treatment with Cetuximab is accompanied by acneiform follicular skin exanthema in more than 80 % of patients. Severe exanthema (grade III/IV) develops in about 9-19 % of patients with the necessity of Cetuximab dose reduction or cessation[39]. Thirdly, Rituximab's property as a highly potent lymphodepleting agent in human subjects is well established, while given its target, no such data exists with Cetuximab; Finally RQR8 is 136 amino acids versus tEGFR's 336 residues.

In summary, we have created a 136 amino acid epitope-based marker/suicide gene for T-cells. The translated protein is stably expressed on the cell surface following retroviral transduction. It binds QBEnd10 analogously to full-length CD34 allowing clinical-grade sorting with off-the-shelf reagents and easy *in vivo* tracking. Further, the construct binds Rituximab; the dual epitope design engenders highly effective CDC and ADCC and consequently renders T-cells highly susceptible to *in vivo* Rituximab mediated depletion. Due to the small size of RQR8, it can easily be co-expressed with a wide range of T-cell engineering components. We anticipate this construct will be of great practical utility and render T-cell therapy safer and ultimately more efficacious.

FIGURE LEGENDS

Figure 1. Engineering of QBEnd10 and Rituximab binding domains

(a) Coding sequences for the 31 extreme amino-terminal residues of mature full-length CD34 (flCD3) were cloned in frame to the CD8 stalk and trans-membrane domain via a serine-glycine linker (LSTK), or without this linker (STK), or connected directly to the CD8 transmembrane domain (flush). These open-reading frames were co-expressed with eGFP within a bicistronic retroviral vector. (b) Flow cytometric analysis of eGFP and QBEnd10 staining in T-cells transduced with flCD34, LSTK, STK. QBEnd10 binding equivalent to that of flCD34 was seen in constructs containing the CD8 stalk, but not with the flush construct. The serine glycine linker did not improve QBEnd10 binding. (c) Further epitope minimisation was performed by sequential amino and carboxy terminal deletion of the 31 residues of CD34 until binding of QBEnd10 was abrogated. In this way, we established a final minimal epitope binding construct containing only 16 amino acid residues from the 385 present in the native antigen. (d) Binding of Rituximab to T-cells transduced with full length-CD20 (CD20), the major extracellular loop of CD20 with some flanking residues on the CD8 stalk (dCD20-loop-v2), the major extracellular loop of CD20 delineated precisely at the constraining cysteine on a CD8 stalk (dCD20-loop-v1), and the circular CD20 mimotope described by Persosa on a CD8 stalk.

Figure 2. Engineering of combination QBEnd10 / rituximab binding constructs

(a) Cartoon of alternative epitope constructs illustrating alternative presentation formats of epitope constructs were generated to enable comparison of sensitivity to CDC and ADCC-mediated deletion: "Q" refers to the 16 amino acid minimized QBEnd10 epitope; "R" refers to the CD20 circular mimotope; "8" refers to the CD8α stalk; Hinge-CH2CH3 refers to the hinge, CH2 and CH3 domains of human IgG1. (b) Binding of QBEnd10 and Rituximab to constructs QR8 and RQ8 co-expressed with eGFP in primary human T-cells is shown. Both antibodies could bind in either orientation. (c) Sensitivity to CDC depletion using primary human T-cells transduced with constructs Q8, RCH2CH3, RQ8, RRQ8, RQR8 and full-length CD20 is shown. Following 4 hour incubation with 25% baby rabbit complement and rituximab at 100μg/ml, samples were stained with Annexin V / PI and the live population was assessed by flow cytometry analysis for presence of the co-expressed eGFP marker gene. Results illustrate comparative deletion observed from 3 separate donors. (d) Similarly, sensitivity to ADCC mediated depletion was assessed using primary human T-cell targets transduced with constructs were challenged by 16:1, 8:1, 4:1 and 2:1 effector:target ratios of NK cell effectors derived from the same donor. Following 48 hour incubation in the presence of 100μg/ml rituximab, depletion was assessed by flow cytometry analysis. Samples were stained with Annexin V / PI for live/dead exclusion, with NK cells labelled with CellTRACE violet excluded from the live gate to identify the residual live population of targets cells identified by the presence of a co-expressed eGFP marker gene. Results illustrate comparative deletion by the 8:1 effector:target ratio observed from 3 separate donors. Construct RQR8 engenders equal CDC and ADCC to full-length CD20.

Figure 3. Functional characterization of RQR8

(a) Demonstration of typical cellular purification result achieved following CD34 magnetic bead selection. Primary human T-cells were transduced with the bicistronic retroviral vector SFG.RQR8.IRES.eGFP and selected with Miltenyi QBEnd10 beads. (b) Efficiency of CDC in transduced sorted T-cells. Primary human T-cells were transduced with SFG.RQR8.IRES.eGFP, purified with QBEnd10 beads and combined at equal concentration with non-transduced T-cells. This mixed population was exposed to a 2 hour incubation with 100μg/ml rituximab and either with or without 25% BRC. T-cells were stained with Annexin/PI and flow-cytometric analysis of eGFP expression on the live population is shown. This is an example of an experiment repeated 6 times in different donors. >95% of the transduced population is deleted. (c) Time-course and Rituximab dose titration assay. CDC mediated deletion of targets was performed in primary human T-cells with rituximab concentrations of 12.5, 25, 50 and 100 μg/ml, analysed at 1, 5, 10, 30, 60 and 120 minutes. Figure shows mean and standard deviation from 3 donors. CDC is highly effective at Rituximab concentrations of 25μg/ml and above and killing occurs within 30 minutes. (d) Demonstration of ADCC mediated sensitivity against T-cells transduced with RQR8. Transduced T-cells were incubated at 16:1, 8:1, 4:1 and 2:1 effector:target ratios of NK cell effectors

derived from the same donor exposed to 100μg/ml rituximab. Samples were stained with Annexin V / PI for live/dead exclusion with depletion assessed by flow cytometry analysis comparison of the ratio of eGFP/eBFP2 marker gene expression from the residual live population. Note: QR8 was not assessed for ADCC sensitivity.

Figure 4. Demonstration of RQR8 activity when co-expressed with a CAR

(a) RQR8 is expressed with an anti-GD2 CAR in the SFG retroviral vector using the foot-and-mouth disease 2A sequence. The retroviral vector expression is enhanced using a scaffold attachment region (SAR). (b) Primary human T-cells were transduced with this retroviral vector. Following transduction, cells were stained with QBEnd10 and polyclonal goat anti-human Fc (the latter stains the CAR). Clear co-expression of RQR8 and CAR was observed. (c) T-cells were sorted with QBEnd10 beads and mixed 1:1 with non-transduced T-cells and exposed to either Rituximab alone or Rituximab and complement. Two hours later, anti-Fc and Annexin V staining was performed. Anti-Fc staining is shown gating on live T-cells. Mean depletion was 97% of transduced cells. (c) This Rituximab depleted population, along with the undepleted T-cells, and non-transduced T-cells were used as effectors in a Chromium release killing assay using GD2+ positive target cells. This experiment was performed in 5 donors.

Figure 5. Binding and killing with Ofatumumab versus Rituxumab.

T-cells well transduced to express either Q8, RQR8 or codon-optimized CD20, all co-expressed with eGFP. (a) T-cells were stained with either Rituximab or Ofatumumab and a secondary anti-human-Fc APC conjugated goat antibody. Antibody binding versus eGFP expression is shown. Q8 binds neither mAbs; CD20 binds both, while RQR8 only binds Rituximab. (b) Next, primary human T-cells were transduced with these constructs and a CDC assay was performed with either Rituximab or Ofatumumab. The percentage lysis as determined by Annexin-V and PI positivity is shown. Q8 transduced to T-cells were not lysed by either mAb; T-cells expressing CD20 were lysed by both, while RQR8 T-cells were lysed only by Rituximab. This experiment was performed in one donor.

Figure 6. In vivo testing of RQR8

C57BL/6 splenocytes were retrovirally transduced to express RQR8 and selected to >90% purity with Miltenyi CD34 beads. 1.5 million of these cells were injected i.v. into 5Gy X-ray preconditioned C57BL/6 x Balb/c cross (F1) recipients. Seven days post transfer engraftment was assessed by peripheral blood. Ritx-mIgG2a Rtx therapy commenced with 150ug doses at day 7, 10 & 12 by i.v. injection or PBS carrier for the control cohort. Each cohort had 5 mice. An additional 2 mice were irradiated but given neither T-cells nor ritux-mIgG2a. Animals were sacrificed at day 14 and engraftment was assessed by considering the proportion of QBEnd10 positive T-cells as a proportion of the T-cell population. (a) Peripheral blood marking levels in control and in treated mice; (b) flow-cytometry of T-cell population in spleen showing RQR8 vs CD8 in mice not receiving transgenic T-cells and control and rituximab treated mice; (c) Percentage marking in lymph-nodes, bone-marrow, blood and spleen. (d) Time-course assessment of rituximab-mediated depletion *in vivo*: C57BL/6 splenocytes were retrovirally transduced to express RQR8 and selected to >90% purity with Miltenyi CD34 beads. 2.0 million cells were injected i.v. into 5Gy X-ray preconditioned C57BL/6 x Balb/c cross (F1) recipients. Five days post transfer engraftment was confirmed by peripheral blood analysis. Ritux-mIgG2a therapy commenced with 150μg doses at day 6, 10 & 12 by i.v. injection with animals sacrificed and assessed for cellular engraftment by considering the proportion of QBEnd10 positive T-cells as a proportion of the T-cell population at 6, 24, 48, 120 and 168 hours following commencement of therapy. Each cohort contained 4 animals. An additional 5 mice were irradiated but given neither T-cells nor ritux-mIgG2a. Percentage marking in spleen, bone-marrow and blood is illustrated for the 6 hour time-point following ritux-mIgG2a therapy.

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AUTHOR CONTRIBUTIONS

MP conceived the idea and designed initial constructs, designed subsequent experiments and wrote the manuscript; BP designed the final construct, co-designed most experiments, performed most of the molecular cloning, retroviral, *in vitro* and *in vivo* work. He assisted in writing the manuscript; LM assisted with in vivo work, ST assisted with molecular cloning, EK assisted with molecular cloning, in vitro and in vivo work; KS assisted with in vitro work. DL designed assisted in design of experiments and writing the manuscript; TM performed the immunohistochemistry. RC, BF, KP and SQ advised and assisted with animal work. VM assisted with some of the molecular cloning;

CONFLICT OF INTEREST DISCLOSURE

Author David Linch is on the Scientific Advisory Board of Cellectis Therapeutics who are licensing RQR8. The laboratories of Martin Pule and Karl Peggs receive funding for contract research from Cellectis Therapeutics.

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