Title: **CRISPR-mediated TCR replacement generates superior anticancer transgenic T-cells**

Running title: TCR replacement for cancer immunotherapy

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- Endogenous TCR knockout increases the expression and functional activity of simultaneously transduced TCR (TCR replacement).
- TCR replacement results in superior targeting of hematological malignancies by T-cells transduced with a non-HLA restricted γδ TCR.

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ABSTRACT

Adoptive transfer of T-cells genetically modified to express a cancer-specific T-cell receptor (TCR) has shown significant therapeutic potential for both hematological and solid tumors. However, a major issue of transducing T-cells with a transgenic TCR is the pre-existing expression of TCRs in the recipient cells. These endogenous TCRs compete with the transgenic TCR for surface expression and allow mixed dimer formation. Mixed dimers, formed by mispairing between the endogenous and transgenic TCRs, may harbor autoreactive specificities. To circumvent these problems, we designed a system where the endogenous TCR-β is knocked out from the recipient cells using CRISPR/Cas9 technology, simultaneously with transduction with a cancer-reactive receptor of choice. This TCR replacement strategy resulted in markedly increased surface expression of transgenic αβ and γδ TCRs, which in turn translated to a stronger, and more polyfunctional, response of engineered T-cells to their target cancer cell lines. Additionally, the TCR+CRISPR modified T-cells were up to a thousandfold more sensitive to antigen than standard TCR-transduced T-cells or conventional model proxy systems used for studying TCR activity. Finally, transduction with a pan-cancer reactive γδ TCR used in conjunction with CRISPR/Cas9 knockout of the endogenous αβ TCR resulted in more efficient redirection of $CD4^+$ and $CD8^+$ T-cells against a panel of established blood cancers and primary, patient-derived B acute lymphoblastic leukemia blasts compared to standard TCR transfer. Our results suggest that TCR transfer combined with genome editing could lead to new improved generations of cancer immunotherapies.

INTRODUCTION

Adoptive transfer of genetically engineered T-cells has become one of the most promising avenues of cancer immunotherapy. Numerous trials have shown objective clinical responses, and even complete remissions, after adoptive cell transfer in patients with cancers resistant to other therapeutic interventions[1–6]. The genetic re-targeting of T-cells to cancer can be achieved either by transduction with a chimeric antigen receptor (CAR) or a T-cell receptor (TCR) specific for an antigen of choice. While CAR-based therapy has proven extremely successful in hematological malignancies positive for CD19 ([7]), CARs can only target surface-expressed molecules. In contrast, use of cancer-specific TCRs allows targeting intracellular proteome and/or metabolome[8].

Vertebrate TCRs exist as heterodimers composed either of αβ or γδ TCR chains. Conventional αβ TCRs recognize short antigenic peptides presented by major histocompatibility complex (MHC) I or II molecules (by $CD8^+$ and $CD4^+$ T-cells, respectively). The targets recognized by human γδ T-cells tend to be predominantly proteins expressed on cell surface in context of a generalized cellular stress, including malignant transformation[9]. A notable exception to this rule is recognition of pyrophosphate metabolites from the mevalonate pathway (henceforth referred to as phosphoantigens) by the predominant peripheral blood subset of γδ T-cells which express TCRs comprised of the Vγ9 and Vδ2 chains[10]. Since there is no evidence for MHC restriction of γδ T-cells, and their targets are expressed on a broad range of cancers, γδ TCRs offer an exciting potential for pan-population immunotherapy[11].

The use of a transgenic TCR in primary, patient-autologous T-cells is hampered by the presence of pre-existing, endogenous TCRs within these cells. Expression of TCR at the cell surface requires the formation of a ternary complex with the CD3 components of this receptor which constitute a limiting factor for surface expression of the antigen-binding chains of the TCR. As a result, successful expression of transduced TCR at the cell surface requires that it must successfully compete with the endogenous TCR chains for CD3 association[12]. In addition, there is also potential for the formation of hybrid TCRs due to mis-pairing of endogenous and transduced TCR chains (so-called mixed TCR dimers). Thus, a transduced T-cell has potential to express four distinct TCRs, only one of which is desired. Mixed TCR dimers can also exhibit unpredictable, and potentially dangerous, target specificities, and have been shown to cause fatal autoimmunity[13].

Several methodologies have been explored to overcome the issue of TCR competition and mispairing. These approaches include generation of affinity-enhanced TCRs[14], engineering of mutations to improve the pairing of transgenic TCRs[15], or overexpression of CD3 components[12]. Affinity-enhanced TCRs have shown high rates of objective clinical response since even a small number of functional TCR molecules is sufficient to convey antigen-specific signaling due to super-physiological activity[16]. However, affinity-enhanced, engineered TCRs have bypassed the rigors of thymic selection and have potential to react to self antigens. Indeed, unanticipated cross-reactivity by an affinity-enhanced MAGE A3-specific TCR with an epitope from titin caused fatal autoreactivity in both patients that were treated with T-cells expressing this TCR[17,18].

Here we aimed to enhance the functionality of *natural* TCRs during TCR gene transfer of primary $CD8^+$ and $CD4^+$ T-cells by simultaneous knockout of the endogenous αβ TCR during transfer of a TCR of choice. This approach enhanced the expression of the transduced TCR at the T-cell surface and resulted in TCR transductants that displayed substantially improved antigen sensitivity. In particular,

we focused on leveraging broadly cancer-reactive γδ TCRs in the TCR transfer system as this approach can be used irrespective of patient HLA type. T-cells transduced with this system were shown to have superior *in vitro* and *ex vivo* reactivity to primary hematological malignancies compared to T-cells expressing both endogenous and transgenic TCRs.

MATERIALS AND METHODS

Cell lines and primary cultures

The following cell lines were purchased from ATCC and cultured according to manufacturer's recommendations: Jurkat E6.1, Molt-3, KBM7, K562, THP-1, U266, TK6. The primary B-acute lymphoblastic leukemia (B-ALL) cells (HP, VB, BV, KÖ, CM, PH) were cultured in defined serum-free media as described previously[19,20]. B lymphoblastoid cell line (LCL) 146 was generated by EBV infection of peripheral mononuclear cells[21] (PBMC) obtained from a healthy donor. Primary B-cells and T-cells were isolated from PBMC based on CD19 or CD4 expression, respectively, and used for functional assays one day after isolation. HLA-A2$^+$ melanoma cell line was cultured in RPMI1640 medium supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine (all from Gibco, Paisley, UK). T-cell clone γδ20 was generated by single cell cloning from PBMC as described[22]. We also made use of an αβ T-cell clone Mel13, specific for a Melan-A epitope EAAGIGILTV presented in context of HLA-A2 ([23]). T-cell clones and lines were expanded in presence of 1 μg/ml phytohaemagglutinin (PHA) and allogeneic irradiated feeders from at least three donors[24].

Generation of transfer vectors and lentiviral particles

TCR from clone γδ20 was sequenced in house using SMARTer RACE kit (Clontech) and two step PCR using universal forward primers and reverse primers specific for constant regions of TCR-γ and TCR-δ. The γδ20 TCR was found to be comprised of a Vγ9 and Vδ2 chain. Mel13 is a sister clone of Mel5 and the TCR sequence has been published before[23] . We have also produced a TCR-peptide-HLA A2 co-complex structure of this TCR with analog[25] and natural[26] antigens. Codon optimized, full length TCR chains, separated by a self-cleaving 2A sequence[27], were synthesized (Genewiz) and cloned into the 3rd generation lentiviral transfer vector pELNS (kindly provided by James Riley, University of Pennsylvania, PA). The pELNS vector contains rat CD2 (rCD2) marker gene separated from the TCR by another self-cleaving 2A sequence. For CRISPR/Cas9 mediated knockout of both TCR-β constant regions (*trbc1* and *trbc2*, IMGT website), four guide RNAs (gRNA) targeting the first exon of *trbc* gene segments were designed using and cloned into pLentiCRISPR v2 plasmid[28] (kindly provided by Feng Zhang, Addgene plasmid 52961). pLentiCRISPR v2 plasmid encodes SpCas9 protein and a puromycin resistance marker gene (pac, puromycin N-acetyltransferase). The sequence alignments of gRNAs are summarised in **Supplementary Figure 1**.

Lentiviral particles were generated by calcium chloride transfection of HEK 293T cells. TCR transfer vectors were co-transfected with packaging and envelope plasmids pMD2.G, pRSV-Rev and pMDLg/pRRE while CRISPR/Cas9 vectors were co-transfected with packaging and envelope plasmids pMD2.G and psPAX2 (all from Addgene). Lentiviral particles were concentrated by ultracentrifugation prior to transduction of T-cells.

T-cell transduction

PBMC were obtained from healthy donors obtained via the Welsh Blood Service. Primary T-cells were purified by Ficoll separation followed by magnetic enrichment for either CD8$^+$ or CD4$^+$ T-cells (Miltenyi Biotec). T-cells were subsequently activated overnight by incubation with CD3/CD28 beads (Dynabeads, Life Technologies) at 3:1 bead:T-cell ratio. After activation the T-cells were transduced with lentiviral particles encoding either only a TCR or both TCR and CRISPR/Cas9, in presence of 5

μg/ml polybrene (Santa Cruz Biotechnology). T-cells that had taken up the virus were selected by incubation with 2 μg/ml puromycin (Life Technologies) and magnetic enrichment with α-rCD2 PE antibody (clone OX-34, Biolegend) followed by α-PE magnetic beads (Miltenyi Biotec). 14 d post transduction T-cells were expanded with allogeneic feeders[22]. For all functional experiments, transduced T-cells were >95% $rCD2^+$.

Flow cytometry

For surface staining, 50,000 cells were stained with Fixable Live/Dead Violet Dye (Life Technologies) and the following antibodies: rCD2 FITC (Biolegend), pan-αβ TCR PE, pan-γδ TCR APC, and CD4 PE-Vio770 and CD8 APC-Vio770 (where applicable; all from Miltenyi Biotec). Mel13 transduced cells were also stained with a cognate tetramer (HLA-A2 refolded in-house with the EAAGIGILTV epitope) according to the optimized tetramer staining protocol[29]. For characterization of the differentiation phenotype of the transduced T-cells, the following antibodies were used: PD-1 PE, CCR7 PerCP-Vio770, CD45RA PE-Vio770, CD45RO FITC, and CD27 APC (all from Miltenyi Biotec). All cell lines tested were stained with BTN3 PE antibody (Biolegend), with or without zoledronate pre-treatment. For Jurkat activation assay, cells were incubated with antigen for 16 h and subsequently stained for CD69. For intracellular cytokine staining, T-cells were incubated for 5 h with target cell lines, and stained for CD107a (BD Biosciences), tumor necrosis factor (TNF)α and interferon (IFN)γ, according to manufacturer's recommendation (all from Miltenyi Biotec). Cells were simultaneous stained for combinations of surface markers rCD2, CD3, CD4 and CD8 as required. Events were acquired on FACS Canto II (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR). Polyfunctionality plots were generated using SPICE software[30]. A minimum of 10,000 viable events were collected per sample.

51-Chromium release assay

For the assessment of cytotoxicity, target cells were pre-incubated with Chromium-51 (Perkin Elmer) and then co-incubated with T-cells at various effector to target (E:T) ratios for 4 h, as described before[29]. Cell lysis was calculated according to the formula below:

$$\%lysis = \frac{experimental\ 51Cr\ release - spontaneous\ 51Cr\ release}{experimental\ 51Cr\ release - maximum\ 51Cr\ release} \times 100\%$$

Enzyme linked immunosorbent assay (ELISA)

Briefly, 30,000 T-cells were co-incubated with 90,000 target cells for 16 h, and the supernatant was harvested. The concentration of macrophage inflammatory protein (MIP)1-β, TNFα or IFNγ in supernatant was quantified using the respective detection kit (R&D Systems), according to manufacturer's instructions. When indicated, target cells were pre-incubated with 50 μM zoledronic acid (Sigma Aldrich) for 16 h and washed extensively before co-incubation with T-cells.

Data analysis

All data were analyzed in GraphPad Prism software, unless specified otherwise.

RESULTS

Design and validation of simultaneous TCR knockout and transfer (TCR replacement) system

Lentiviral transduction of primary T-cells is greatly enhanced when the cells are actively dividing in response to TCR and co-stimulatory signals[31]. To incorporate this enhancement and produce a simple, time-efficient methodology that could be applied with many existing TCR transduction systems we activated T-cells in the presence of two separate lentiviral populations, one encoding the TCR of choice as a transgene, the other CRISPR/Cas9 targeting the endogenous (but not codon-optimized) TCR-β constant region (*trbc1* and *trbc2*) as described in Materials and Methods. Four guide RNAs (gRNAs) targeting TCR-β were designed and showed >90% knockout efficiency in Jurkat T-cell leukemia line (**Supplementary Figure 1A**). gRNA 1 was selected for use in primary T-cells due to the high degree of mismatch between endogenous and the standard, codon-optimized TCR-β sequences generally used during TCR transduction (**Supplementary Figure 1B**). The TCR and CRISPR lentiviruses encoded two different selection markers (ectopically expressed rat CD2 and puromycin resistance gene, respectively; **Figure 1A**) allowing selection of cells that had integrated the lentiviral cargo. In addition, the use of rat CD2 which was stoichiometrically expressed with the TCR allowed ready comparison between different donors and different transduction conditions (TCR only or TCR+CRISPR). Following lentiviral transduction, transduced cells were selected by magnetic or fluorescence-based sorting and culturing with puromycin, where applicable, followed by conventional T-cell expansion protocol (**Figure 1B**). While the selection of transduced cells by rat CD2-based purification and puromycin treatment resulted in nearly 90% decrease in cell number (**Supplementary Figure 2A**), the selected cells were then capable of expanding to the same extent as untransduced cells for at least five consecutive expansions with allogeneic feeders and PHA (**Supplementary Figure 2B**). Notably, transduction efficiency with TCR-bearing lentivirus was decreased in presence of CRISPR lentivirus, indicating that a fraction of cells were capable of accepting only one of lentiviruses (**Supplementary Figure 2C**).

Transduction of primary αβ T-cells with a γδ TCR on its own resulted only in a minor downregulation of the endogenous αβ TCR expression. In contrast, αβ TCR expression was almost completely ablated when cells were co-transduced with TCR and CRISPR/Cas9 in all donors tested, showing high efficiency of the TCR replacement system (**Figure 1C**). We then compared the expression of pyrophosphate metabolite specific γδ TCR (γδ20), or a melanoma antigen specific αβ TCR (Mel13) in primary $CD8^+$ αβ T-cells which were either single (TCR) or double (TCR+CRISPR) transduced. While the expression of transgenic TCRs in single transduced T-cells was relatively low (as detected by pan-γδ TCR antibody or a cognate Mel13 tetramer, HLA-A2:EAAGIGILTV), co-transduction with the CRISPR vector resulted in dramatically enhanced expression of the transduced TCR in all donors tested (up to 10-fold increase in mean fluorescence intensity, as well as a distinct shift of histogram peak; **Figure 2**). High level of expression of the transgenic αβ TCR in double transduced cells further confirmed that TCR-β targeting gRNA was unable to cleave the codon optimized receptor, and that the presence of endogenous TCRα chains did not have a detrimental effect on the expression of the transgenic TCR.

TCR replacement improves the functional response of transgenic T-cells to target cells

It is generally acknowledged that the number of functional TCR molecules on the surface of a T-cell is one of the factors governing T-cell sensitivity to an antigen[32]. Therefore, we activated the single or

double transduced $CD8^+$ T-cells with their target cell lines (B-LCL line pre-incubated with zoledronate for γδ20 TCR, and a HLA-$A2^+$ melanoma cell line for Mel13 TCR), and measured the percentage of cells expressing a marker of cytotoxicity CD107a, and two cytokines interferon (IFN)γ and tumor necrosis factor (TNF)α. The response of TCR+CRISPR transduced T-cells was markedly stronger than that of cells transduced with only TCR (**Figure 3A** and **Supplementary Figure 3**). Up to 90% of TCR+CRISPR transduced cells expressed at least one activation marker in response to target cells, and most of these cells expressed all three markers tested for, indicating a strong, polyfunctional response to antigen. Conversely, less than 10% and 40% of cells transduced only with γδ20 and Mel13 TCR respectively were capable of mounting a response to the target cells - with only a small fraction of the cells that did respond exhibiting more than one function. In comparison, TCR transduced cells with CRISPR knockout were capable of mounting a statistically significantly stronger response to their cognate antigen in all donors tested (**Figure 3B**), and the response of TCR+CRISPR cells was comparable to that of parental clones. Both TCR only and TCR+CRISPR cells were capable of downregulating the transgenic TCR upon stimulation with the cognate antigen (**Supplementary Figure 4**). Importantly, the untransduced and single/double transduced cells showed similar terminally differentiated effector memory phenotype[33], plausibly resulting from CD3/CD28 bead expansion, but no signs of T-cell exhaustion, in terms of PD-1 expression[34] (**Supplementary Figure 5**).

TCR replacement improves the sensitivity to antigen of a γδ TCR by several orders of magnitude

T-cells require a given copy number of antigen to be present on target cells in order to mount a successful response, thus defining the antigen sensitivity. While antigen sensitivity may be manipulated in case of αβ T-cells by affinity maturation of the TCR[14] so that it can robustly respond to a very limited number of antigen copies[35,36], no such technology has been developed for γδ TCRs. Therefore, we decided to investigate if increasing the copy number of γδ TCR on transgenic T-cells by CRISPR/Cas9 knockout of endogenous TCRs would increase the sensitivity to the cognate antigen HMBPP ((E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate) by TCR-transduced cells. In parallel we tested the model proxy system for studying the role of TCR in target cell recognition; namely the Jurkat T-cell leukemia line[9]. We measured T-cell response to the antigen in terms of MIP-1β secretion (for T-cells) or CD69 upregulation (Jurkat). In our experience, MIP-1β secretion has been the most sensitive methods for detecting T-cell activation[35–41]. Indeed, when we used IFNγ as a readout of T-cell activation, we could not detect any meaningful response from γδ20 TCR-only transduced T-cells while the activation of TCR+CRISPR cells closely replicated that of the parental clone (**Supplementary Figure 6A)**. Furthermore, the parental T-cell clone was more sensitive to the antigen by four orders of magnitude (**Figure 4**) than the Jurkat cell line (when using MIP-1β and CD69 as markers of activation, respectively). More importantly, TCR-only transduced $CD8^+$ cells were only slightly more sensitive than Jurkat cell line, and well over a thousandfold less sensitive than the T-cell clone. In contrast, TCR+CRISPR transduced cells showed a similar degree of antigen sensitivity as the parental clone, and were >50,000 or >5,000 more sensitive than Jurkat or single transduced T-cells, respectively. The improvement in antigen sensitivity observed with Mel13 TCR+CRISPR transduced T-cells compared to Mel 13 TCR-only transduced T-cells was more modest than for γδ20 TCR (~10-fold greater sensitivity in terms of MIP-1β production; **Supplementary Figure 6B and C)**. Importantly, the improvement observed with Mel13 TCR+CRISPR T-cells extended to superior cytotoxic activity against HLA-$A2^+$ melanoma targets compared to cells transduced with Mel13 TCR only (**Supplementary Figure 7)**.

Endogenous TCR knockout enhances recognition of hematological malignancies *via* a γδ TCR

Vγ9Vδ2 TCRs are known to recognize metabolites of the mevalonate pathway in context of butyrophilin 3A1 molecule[42–44]. The mevalonate pathway is often dysregulated in cancer cells[45], and can be further modulated by aminobisphosphonates such as clinically approved zoledronate[46]. Vγ9Vδ2 T-cells and TCRs thus have the potential to target multiple different cancer types. Therefore, we first tested the cytotoxic activity of γδ20 TCR-transduced cells against an LCL line derived from the same donor as the parental T-cell clone. In line with the polyfunctionality profile described above (**Figure 3**) TCR+CRISPR transduced $CD8^+$ cells were able to exhibit stronger cytotoxic activity, especially at low effector:target (E:T) ratios (26% *vs.* 4% at 0.8 E:T, **Figure 5A**) to LCL pre-incubated with zoledronate. No cytotoxicity was observed without zoledronate pre-treatment, even in case of the parental T-cell clone, thus indicating that the endogenous accumulation of mevalonate metabolites in that cell line was not sufficient to trigger T-cell activation.

Since Vγ9Vδ2 T-cells do not require CD8/CD4 co-receptors for target recognition, we then investigated the potential of TCR replacement system to redirect both $CD8^+$ and $CD4^+$ T-cell subsets to a panel of hematological malignancies. We tested the ability of single (γδ20 only) and double transduced (γδ20 + CRISPR) T-cells to undergo activation and cytokine secretion (TNFα and IFNγ) in response to established blood cancer cell lines (T ALL, acute myeloid leukemia (AML), multiple myeloma) as well as primary, patient-derived B ALL cells (**Figure 5B, Supplementary Figure 8**). The primary B ALL cells used here have been previously shown to closely replicate the characteristics of the cancer without cell culture induced bias[19,20]. Single transduced T-cells showed only low reactivity, or no reactivity at all (especially in case of Molt3 T ALL line and primary B ALL cells) to hematological malignancies pre-treated with zoledronate; conversely, TCR+CRISPR transduced cells responded to all cell lines tested, in a much stronger manner than the TCR only transduced cells, even to cancer cells expressing an almost undetectable level of BTN3 on the surface (**Supplementary Figure 9A)**. No reactivity of γδ20 TCR transduced cells (with or without TCR-β CRISPR) was observed against freshly isolated, zoledronate-treated healthy cells (**Figure 6**), despite strong BTN3 expression on the cell surface (**Supplementary Figure 9B**).

DISCUSSION

TCR gene transfer has been proven as a clinically successful means of redirecting patient's immune system to combat different cancer types[47]. However, the pre-existence of endogenous αβ TCRs in the recipient T-cells has limited the clinical use to highly competitive/high affinity αβ TCRs. Here we demonstrate that cancer-specific αβ or γδ TCRs that do not compete well with recipient TCRs, and therefore exhibit weak functional activity, can be efficiently used to redirect recipient T-cells to cancer if combined with simultaneous knockout of endogenous TCR-β. The resultant engineered T-cells were as sensitive to antigen as the starting T-cell clone suggesting that mispairing between endogenous TCR-α chains and transduced TCR-β must be minimal. This finding is in accordance with the results of Provasi *et al.* using zinc finger nucleases where transgenic TCR activity was comparable in T-cells deficient for only the endogenous TCR-β and both TCR-α and –β[48]. Furthermore, since TCR-α and –β chains cannot pair with TCR-γ and –δ chains, disruption of just TCR-β chain is sufficient to achieve the optimal expression of transgenic γδ TCRs.

To date, there have been several reported attempts to combine endogenous TCR knockout, using zinc finger nucleases[48,49], transcription activator-like effector nucleases[50–53] or CRISPR/Cas9[54], with redirecting the T-cells to cancer, in most cases *via* CARs. This is the first report demonstrating successful redirection of primary T-cells with a pan-cancer reactive γδ TCR in combination with endogenous TCR-β knockout. We showed that removal of the endogenous TCR-β chain leads to a striking increase of surface expression of transgenic αβ and γδ TCRs that translates into a much stronger response of engineered T-cells to cancer lines. While it has recently been shown by Eyquem *et al.* that CAR insertion into the TCR locus is beneficial due to limiting and controlling CAR expression by physiological means, thus preventing premature exhaustion, the antigen binding kinetics and affinity of natural TCRs differ significantly from that of CARs, and therefore high copy number of TCRs on the cell surface appears more desirable[55]. Indeed, CRISPR+TCR transduced T-cells exhibited a significantly more polyfunctional response profile when presented with target cells than that observed with TCR-transduction in the absence of TCR-β knockout, without any apparent changes in terms of T-cell differentiation and exhaustion. Importantly, Ding *et al.* showed that polyfunctional T-cells are crucial for achieving a successful clinical outcome in patients suffering from hematological malignancies[56]. Moreover, this is the first side-by-side comparison of the antigen sensitivity of model Jurkat T-cell line, primary T-cells transduced with a given TCR and the parental T-cell clone using the most sensitive readouts available. Our results indicate that the antigen sensitivity of model systems used in research such as Jurkat cells, or in the clinic (primary T-cells) are up to several orders of magnitude lower than that of the parental T-cell clone, and that the sensitivity of the latter can be accurately replicated by combining TCR transfer with endogenous TCR knockout (TCR replacement) in primary T-cells. Apart from having implications for designing more effective TCR-based immunotherapies, this result indicates that TCR *replacement* is preferable to TCR *transfer* for functional characterizations of TCRs of interest especially where these TCRs compete poorly with endogenous TCRs for surface expression or have a relatively low affinity for cognate antigen. This approach should also enable detailed analysis of TCR recognition in the absence of parental T-cell clones. Such TCRs may come from T-cells that display poor growth characteristics (e.g. as a result of cancer-mediated T-cell exhaustion[57]), or directly from high throughput sequencing of TCR repertoires[58]. One can also envisage that primary T-cells transduced with a TCR of unknown specificity but not expressing the endogenous TCRs could be used for high-throughput, whole-genome screens[28] to identify new TCR ligands, and therefore new potential therapeutic targets.

γδ T-cells offer an attractive tool for cancer immunotherapy, due to their ability to recognize ubiquitously expressed targets and no evidence of MHC restriction. This feature allows such γδ T-cells to respond to cancer from any individual and also eliminates the risk of graft *versus* host disease[59]. To date, the majority of clinical trials utilizing γδ T-cells have focused on the predominant subset in the periphery, namely Vγ9Vδ2 T-cells, which respond to phosphoantigen metabolites. Several multi-center clinical trials[60–63] have demonstrated that *in vivo* activation of Vγ9Vδ2 T-cells and cancer cell sensitization with aminobisphosphonates (zoledronate, pamidronate) was well tolerated and did not result in off-target toxicities (despite the ubiquitous expression of butyrophilin molecules and mevalonate pathway components). Encouragingly, aminobisphosphonate treatment resulted in objective clinical responses in a fraction of patients with non-Hodgkin lymphoma, multiple myeloma[60] and AML[62] demonstrating the potential of γδ T-cell based immunotherapies for hematological malignancies. However, the therapeutic success of γδ T-cell immunotherapies remains underwhelming[11], especially compared to CD19-CAR therapies[64]. One of the potential reasons for this poor success could be the use of variable and largely undefined (especially in terms of TCR usage) cellular product. Furthermore, antigen-driven expansion of Vγ9Vδ2 T-cells, as used so far, has been shown to lead to exhaustion and loss of functional activity, in both animal models and in patients[65,66]. In contrast, TCR replacement by gene transfer, as utilized here, could be applied to a desirable T-cell subset (for instance, T memory stem cells) thereby potentially allowing improved host engraftment and/or function[67]. We propose that using a defined γδ TCR transferred to patient's T-cells in combination with the knockout of endogenous αβ TCRs could be a therapeutically beneficial strategy. Indeed, TCR+CRISPR T-cells showed a markedly stronger response (in terms of TNFα and IFNγ production) than TCR-only transduced T-cells to established cancer cell lines, as well as all primary B-ALL blasts. It should be noted that TNFα production was shown to correlate with cancer-specific activity of cytotoxic T-cells, and elevated intratumoral TNFα concentration could serve as a favorable prognostic factor[68]. Similarly, IFNγ is a potent immunomodulatory cytokine that enhances T-cell mediated recognition of cancer cells and plays a direct anticancer role[69,70]. No on-target off-tumor reactivity of γδ20 TCR transduced cells was observed against primary, aminobisphosphonate-treated B-cells, T-cells or whole PBMC, in line with multiple clinical trials indicating that aminobisphosphonate treatment (with or without infusion of *ex vivo* expanded γδ T-cells) was generally well tolerated without severe adverse effects (reviewed in [11]). It should be noted, however, that even with using the TCR replacement technology for generation of Vγ9Vδ2 TCR transgenic T-cells, the patients will most likely require bisphosphonate treatment for efficient cancer sensitization, in addition to adoptive transfer of TCR engineered T-cells. The increasing clinical experience in co-administering aminobisphosphonates and Vγ9Vδ2 T-cells[62,71–74] will undoubtedly facilitate designing of clinical trials testing the efficacy of Vγ9Vδ2 TCR transgenic T-cells. We therefore believe that the TCR replacement technology described here would be of use in fundamental and translational research where it could, for instance, be utilized to discover ligands of clinically relevant TCRs. In addition, this kind of approach has potential for developing a new generation of TCR-based immunotherapies, provided the method is optimized for the clinical scale, based on the wealth of experience in generating therapeutic CAR T-cells. Widespread clinical application of gene editing technology seems imminent, as demonstrated by the recent success of the off-the-shelf allogeneic CAR19 T-cells in inducing remission of B-ALL in infants[53]. In summary, it seems likely that TCR replacement by CRISPR/Cas9, or other means, will generate clinically useful T-cells that do not encompass the dangers of TCR mispairing and that can be orders of magnitude more sensitive than the products currently being trialed.

AUTHORSHIP

Contribution: M.L., G.D. and A.K.S. conceived the study, designed the experiments and wrote the manuscript. M.L. performed the experiments and analyzed the data. A.M. and O.O. provided the reagents and guidance.

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FIGURE LEGENDS

Figure 1. Construct design and validation for transduction of primary T-cells. (**A**) Schematic representation of transgenes cloned into pELNS vector (top) or lentiCRISPRv2 vector (bottom). EF-1α - elongation factor-1 alpha promoter, U6 - RNA polymerase III promoter, pac - puromycin N-acetyltransferase, EFS - short EF-1α promoter. (**B**) Timeline for transduction and selection of primary T-cells. (**C**) Graphical representation of TCR expression on primary T-cells transduced with pELNS vector, with and without lentiCRISPRv2 vector (top). Grey molecules represent endogenous TCR chains while blue ones represent transduced TCR chains. The histograms below show endogenous αβ TCR expression in three donors (grey – untransduced, blue – transduced only with a γδ TCR, red – transduced with a γδ TCR and CRISPR), as well as a representative unstained control (black). The color coding is maintained throughout the manuscript. The numbers on histograms refer to geometric mean fluorescence intensities of αβ TCR expression across three donors (D1, D2, D3).

Figure 2. **The expression of transduced TCRs in primary CD8$^+$ αβ T-cells derived from three healthy donor PBMC is markedly increased in presence of CRISPR/Cas9 specific for endogenous TCR-β**. Histograms represent staining of transduced CD8$^+$ cells with a pan-γδ TCR antibody (left) or with a HLA-A2:EAAGIGILTV tetramer cognate for Mel13 TCR (right), while the numbers refer to geometric mean intensity of staining. Unstained control is shown in black while grey represents untransduced T-cells, blue – transduced only with a TCR, red – transduced with a TCR and CRISPR.

Figure 3. The functional response to target cell lines is significantly increased in CD8$^+$ T-cells co-transduced with TCR and CRISPR/Cas9 specific for endogenous TCR-β. (**A**) Polyfunctionality plots representing the response of transduced and untransduced T-cells in comparison to the parental T-cell clone. Top row shows the response to a B LCL line pre-incubated with zoledronate by cells transduced with the γδ20 TCR. Bottom row shows responses to an HLA-A2$^+$ melanoma cell line by cells transduced with the Mel13 $\alpha\beta$ TCR. Only viable CD3$^+$ cells were included in the analysis while the gates for cells positive for a given function were set based on appropriate fluorescence minus one and biological controls. Representative data from two independent experiments and three donors are shown. (**B**) The response of transduced T-cells to target cell lines, in terms of CD107a, IFNγ and TNFα expression (mean and standard deviation from three donors are shown). The percentage of cells that were positive for a given function in absence of cognate stimulus (i.e. T-cells + B LCL for γδ20 TCR, and T-cells alone for Mel13 TCR) was subtracted from the percentage of cells positive in the presence of cognate stimulus (i.e. T-cells + B LCL pre-incubated with zoledronate or T-cells + HLA-A2$^+$ melanoma cell line, respectively). The statistical significance of difference between the response of cells transduced only with TCR or with TCR + CRISPR was measured by paired Student t-test. ***p=0.0001, **p=0.002

Figure 4. **The sensitivity to antigen of γδ20 TCR + CRISPR transduced CD8$^+$ cells is higher by several orders of magnitude than the sensitivity of CD8$^+$ cells transduced only with γδ20 TCR**. (**A**) The sensitivity to the titrated antigen HMBPP was measured either by CD69 mobilization (Jurkat) or MIP-1β production (transduced T-cells and T-cell clone) after overnight incubation with the antigen. CD69 mean fluorescence intensity or MIP-1β concentration were normalized by subtracting the values of unstimulated cells, and assuming the maximum value as 100%. The EC_{50} values were calculated in GraphPad Prism software by non-linear regression curve fitting. (**B**) EC_{50}, represented as molar

concentration of antigen and fold change. Representative data of two independent experiments carried out in duplicate are shown.

Figure 5. T-cells transduced with CRISPR replacement show a markedly stronger response to blood cancer lines than with standard transduction techniques. (**A**) 4 h cytotoxicity of transduced $CD8^+$ cells, as well as parental γδ20 T-cell clone, against an untreated (empty symbols) or zoledronate-pretreated (filled symbols) γδ20 donor-autologous B LCL. Representative data are shown from three donors tested in two experiments carried out in duplicate. (**B**) TNFα secretion by transduced $CD8^+$ (top) or $CD4^+$ (bottom) T-cells after overnight co-incubation with a panel of established blood cancer lines of diverse lymphoid and myeloid origin, or patient-derived B ALL cells. Cancer cells were pre-incubated with zoledronate for 24 h before co-incubation with T-cells. TNFα secretion was normalized by subtracting TNFα produced by T-cells alone, and by cancer cells alone. No specific TNFα secretion by T-cells was observed in absence of zoledronate pre-treatment. Representative data are shown from three donors and two experiments carried out in duplicate.

Figure 6. Increase of γδ TCR expression by TCR-β knock-out does not enhance the targeting of normal cells by engineered T-cells. Normal cells were isolated from peripheral blood of three healthy donors (PBMC isolation followed by magnetic pullout of $CD19^+$ B-cells or $CD4^+$ T-cells) and incubated with 50 μM zoledronate (where indicated). On the following day after isolation, the cells were co-incubated with transduced T-cells for 16 h, followed by quantification of secreted TNFα or IFNγ. The concentration of secreted cytokines was normalized by subtracting the values from T-cells incubated alone and target normal cell incubated alone. Leukemia cell line THP1 and myeloma cell line U266 were included as positive controls. Representative data from two TCR-transduced donors are shown.

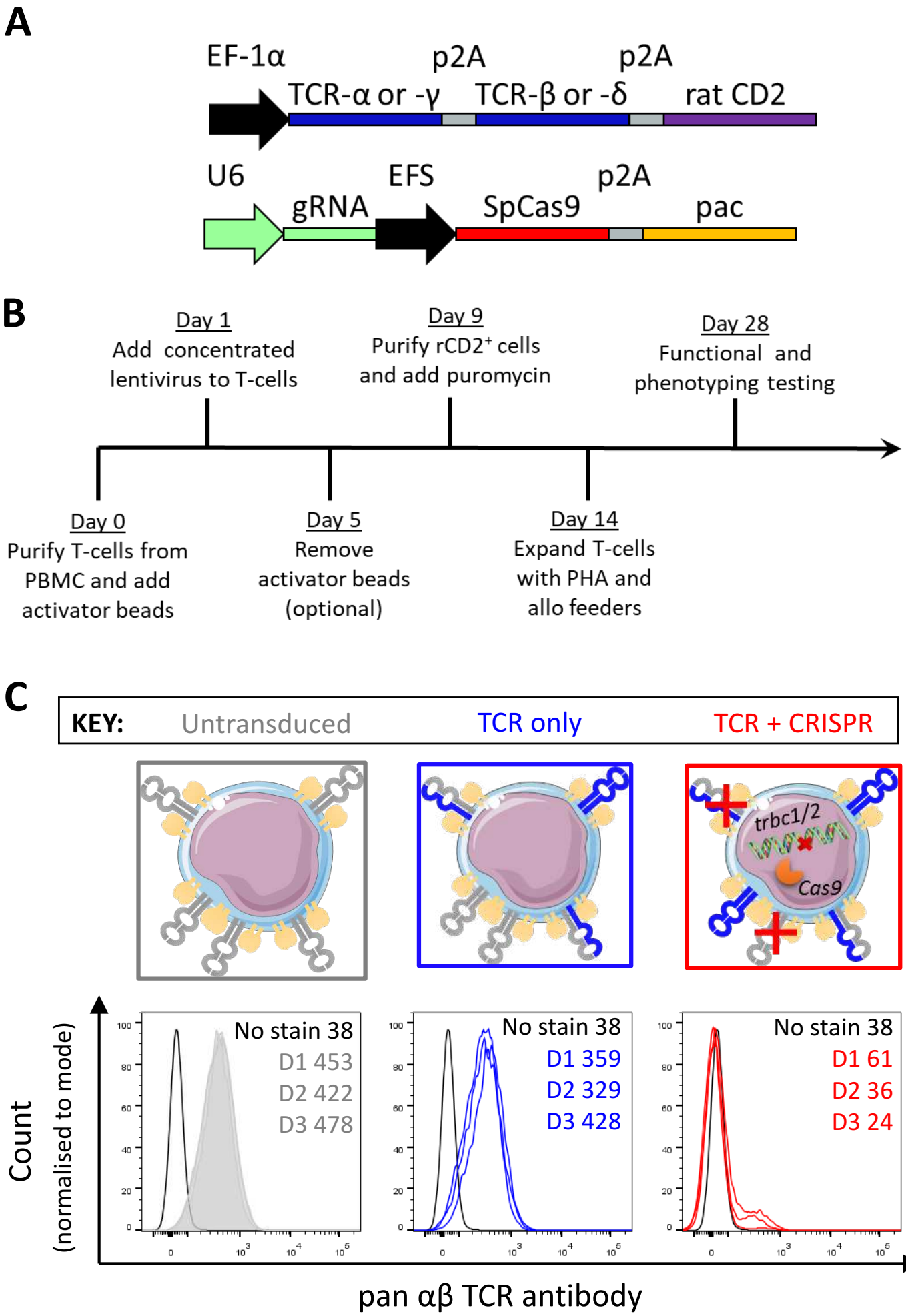
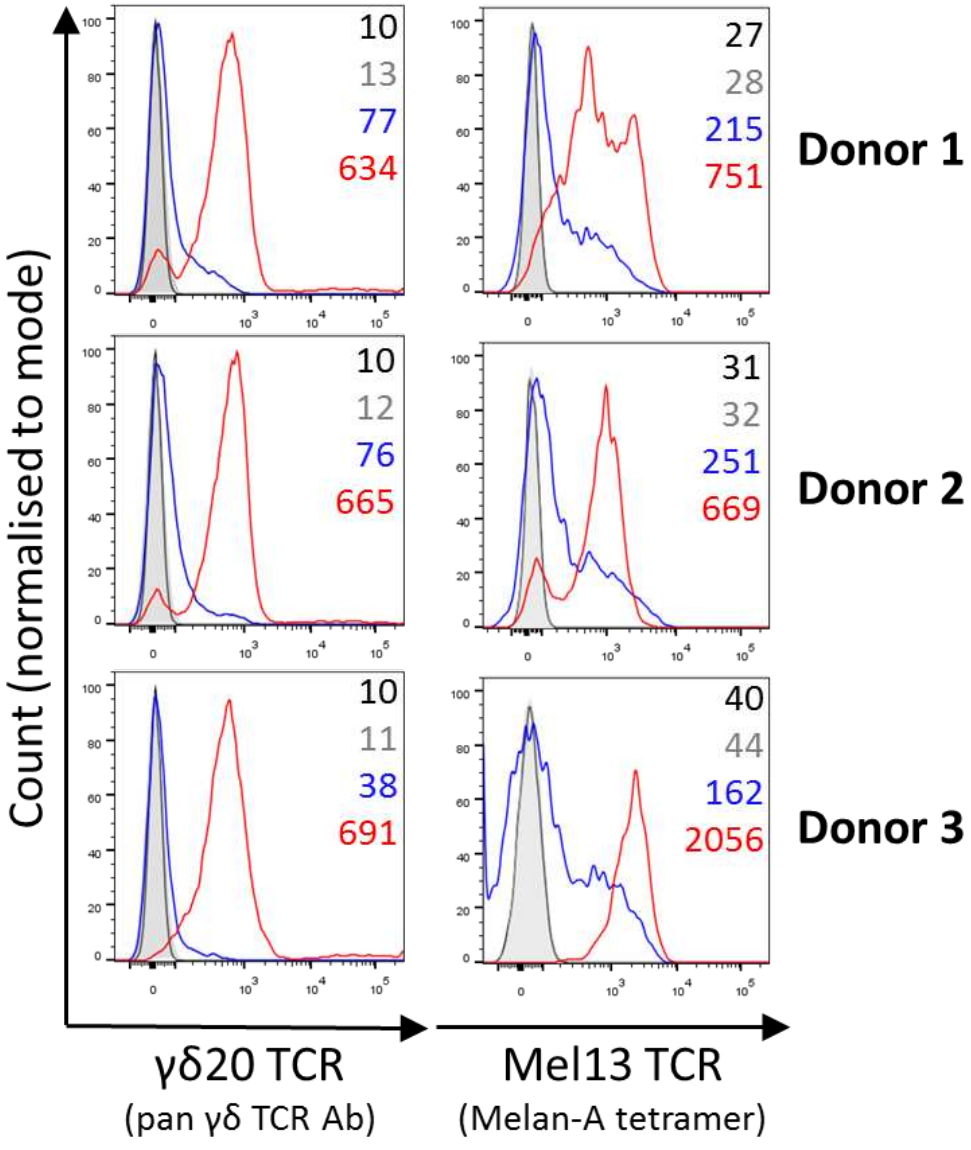


FIGURE 1 LEGUT ET AL.



KEY: No stain Untransduced TCR only TCR + CRISPR

FIGURE 2 LEGUT ET AL.

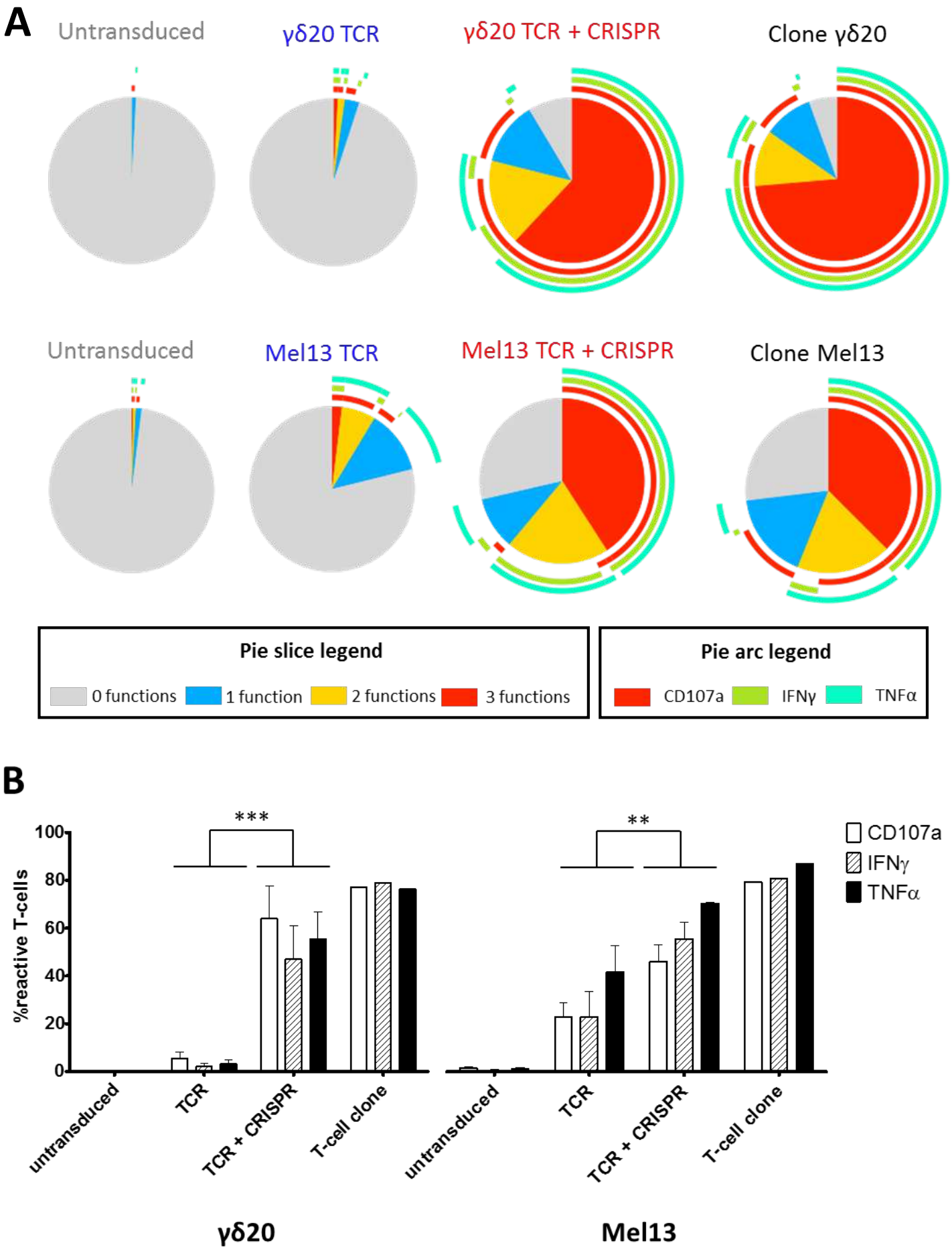
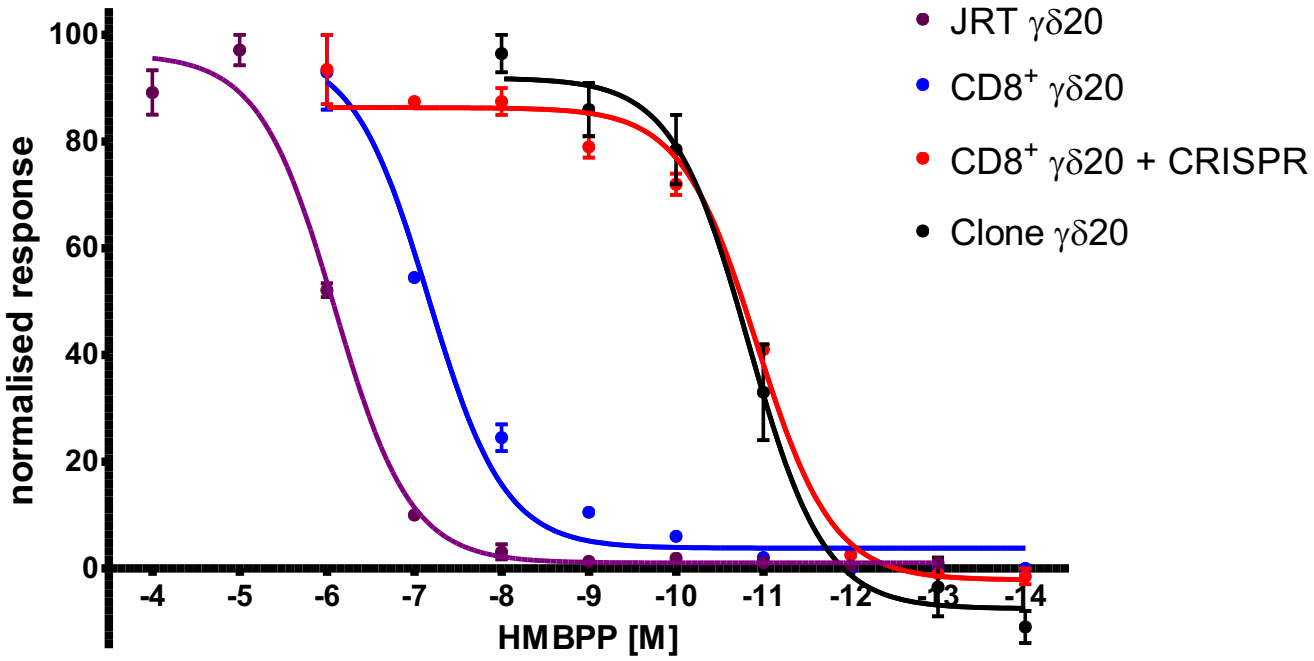


FIGURE 3 LEGUT ET AL.

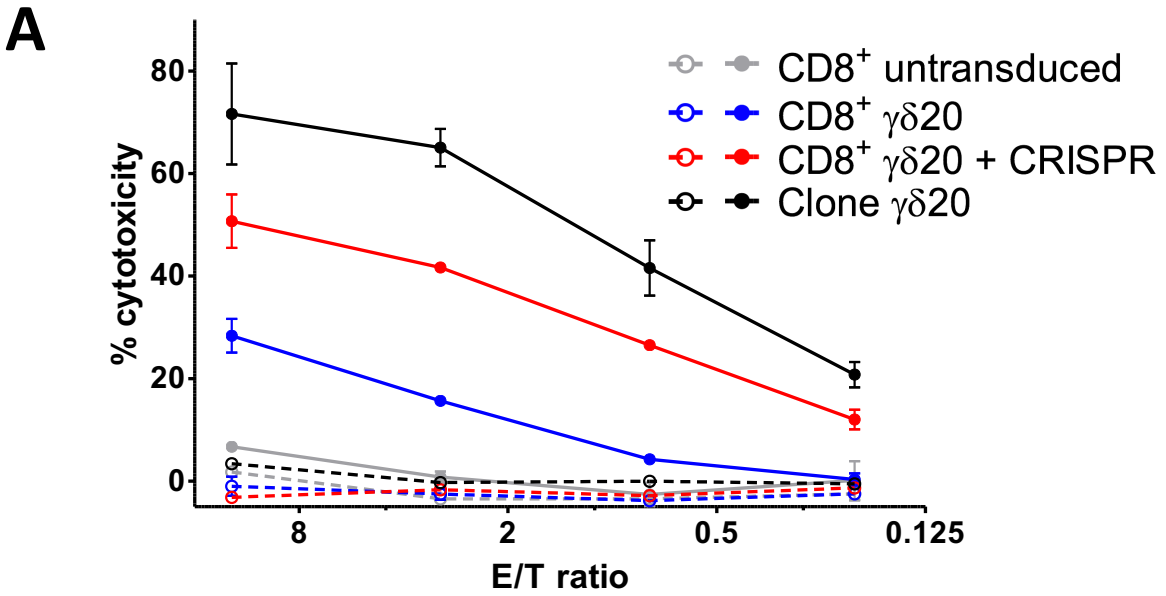
A



B

Sample	EC_{50} [M]	Fold change of EC_{50}
JRT γδ20	8.1×10^{-7}	0.1
CD8+ γδ20	6.8×10^{-8}	1
CD8+ γδ20 + CRISPR	1.2×10^{-11}	5,000
γδ20 clone	1.5×10^{-11}	5,000

FIGURE 4 LEGUT ET AL.



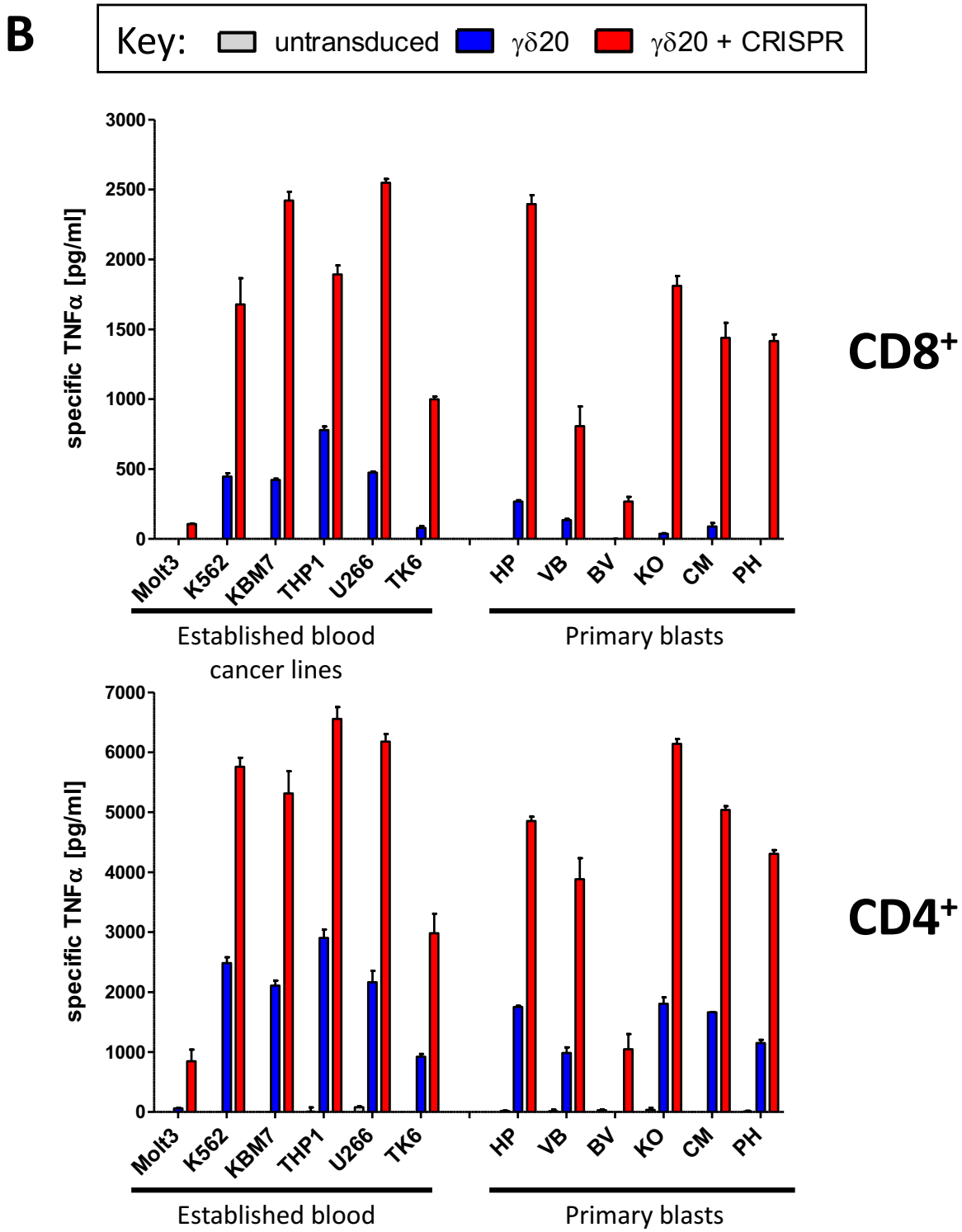


FIGURE 5 LEGUT ET AL.

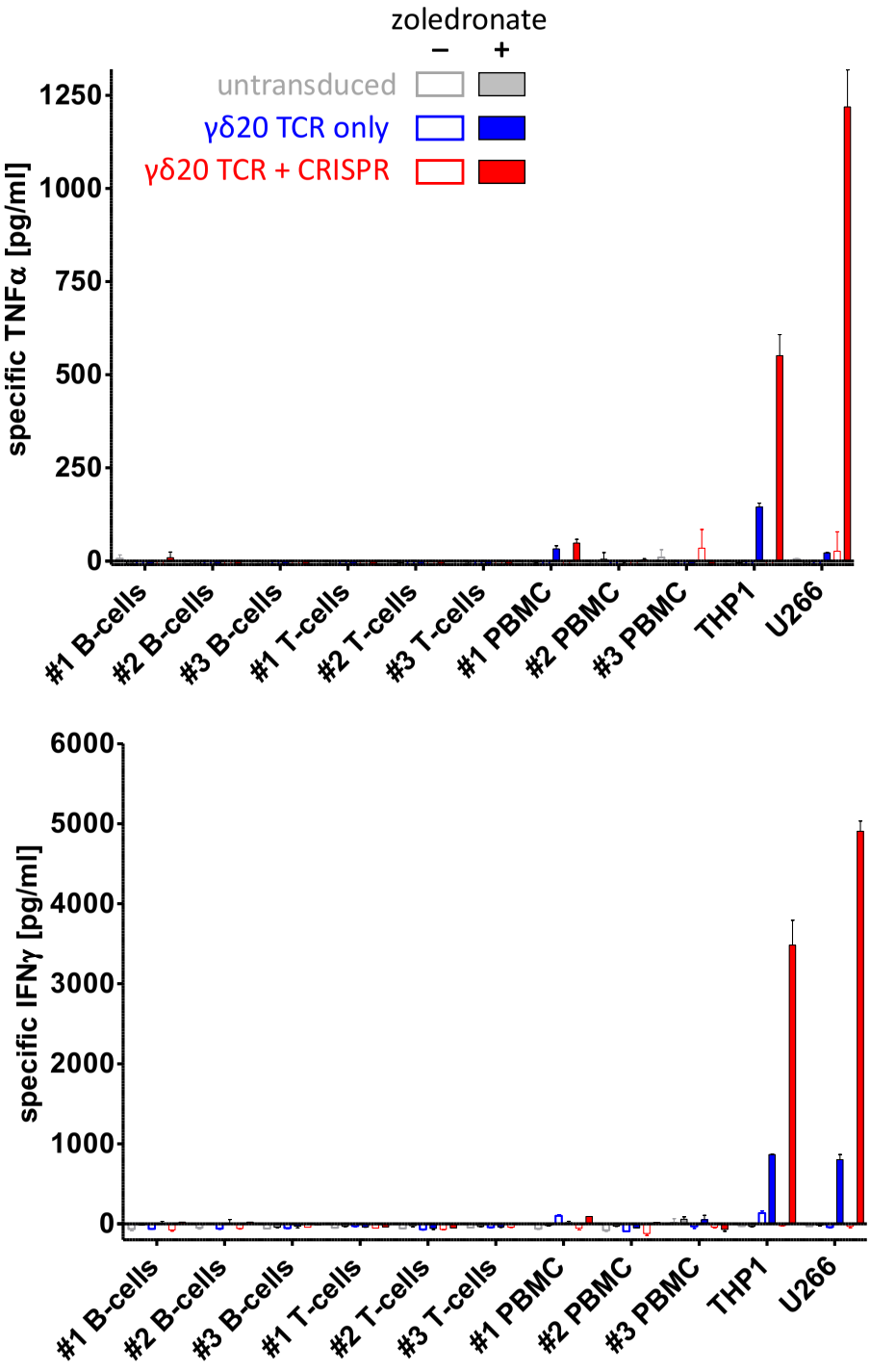


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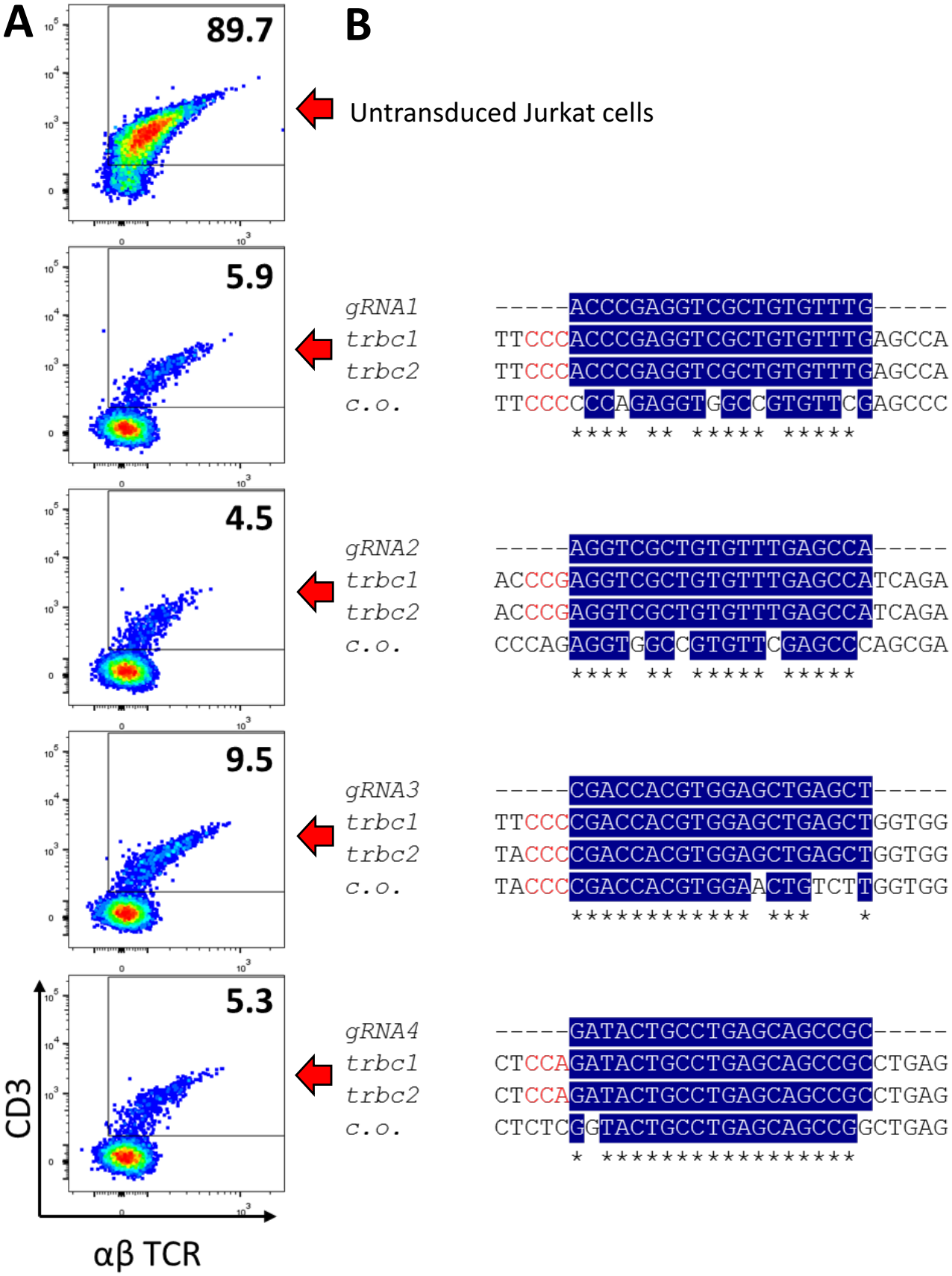
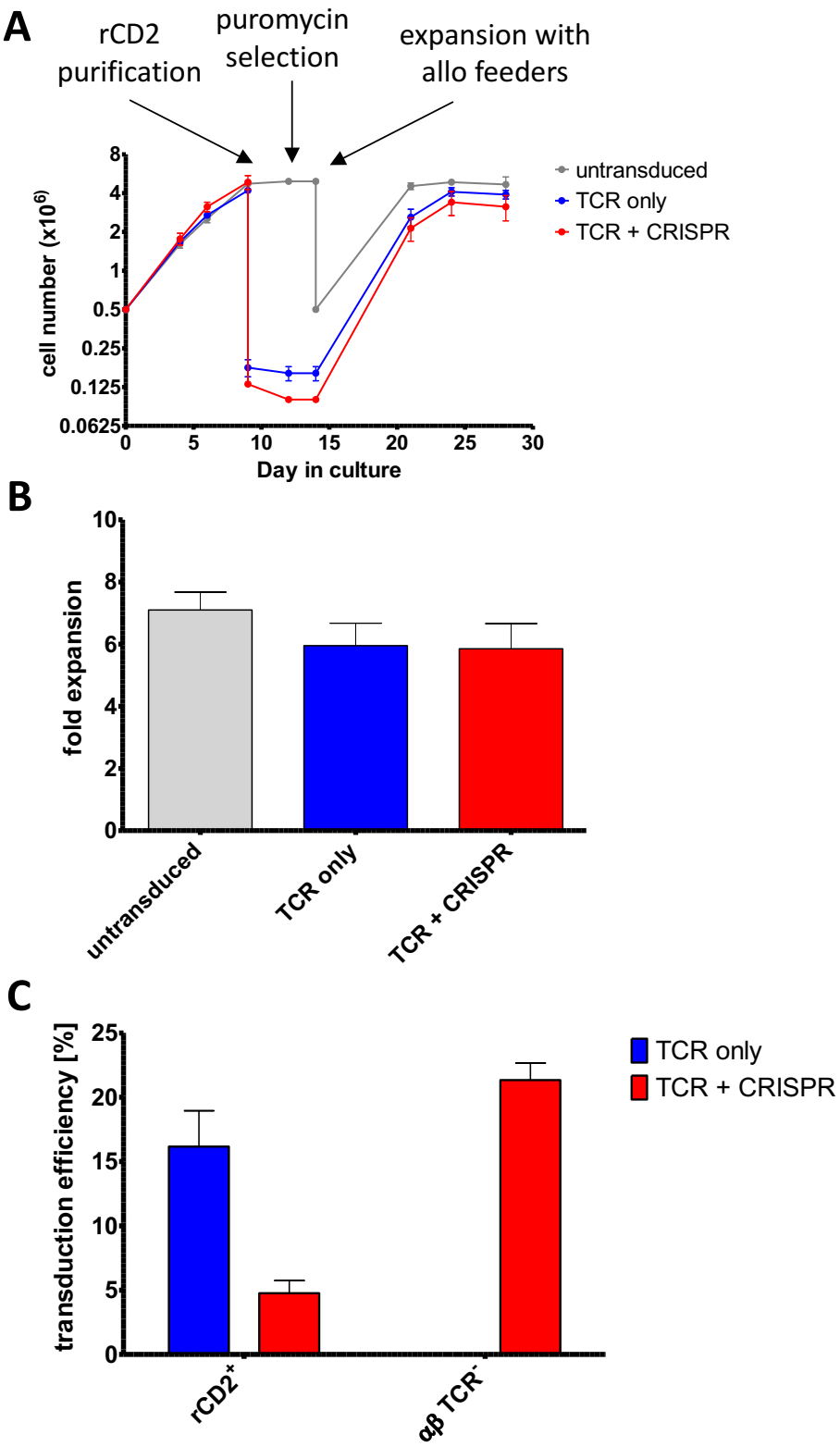
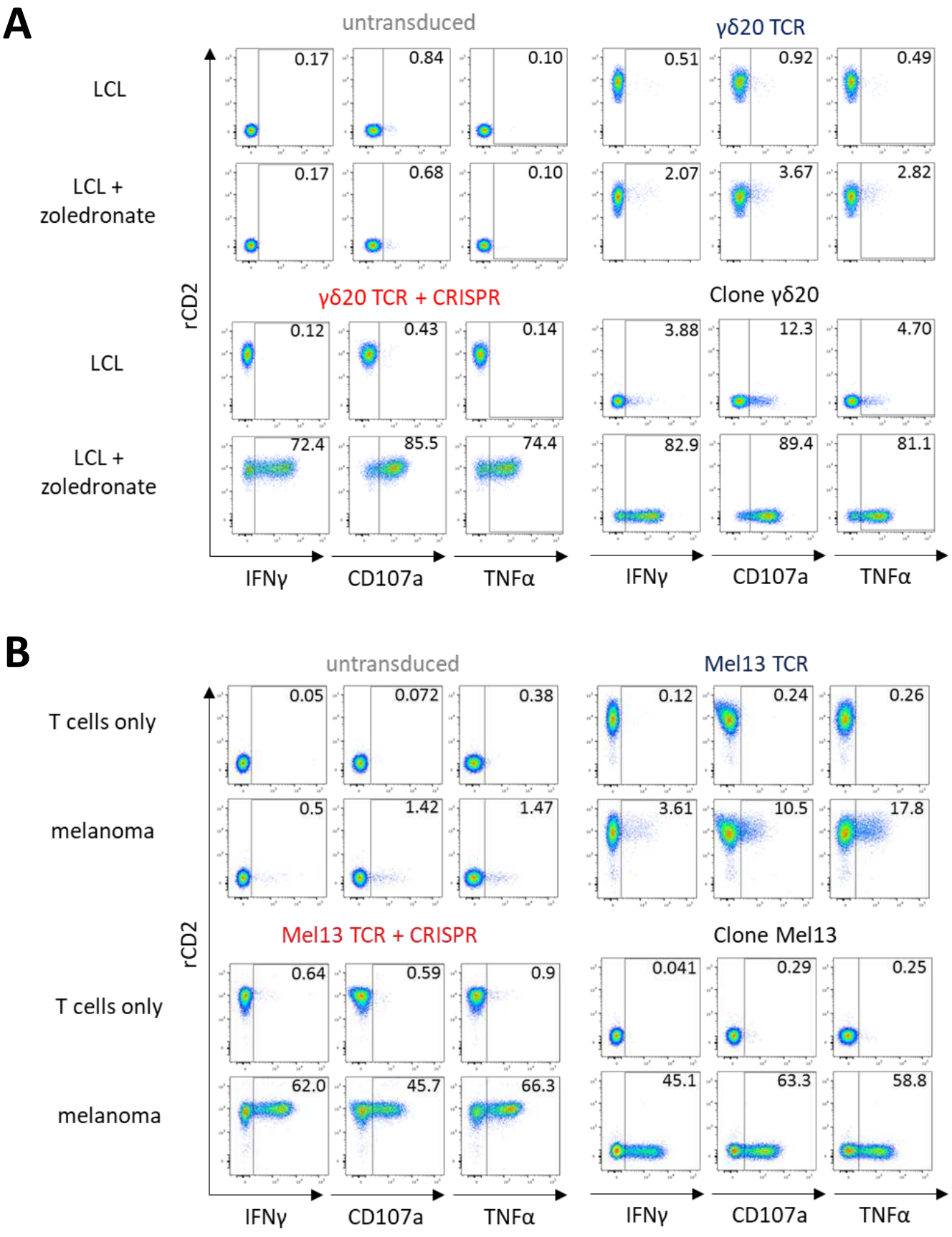
FIGURE 6 LEGUT ET AL.



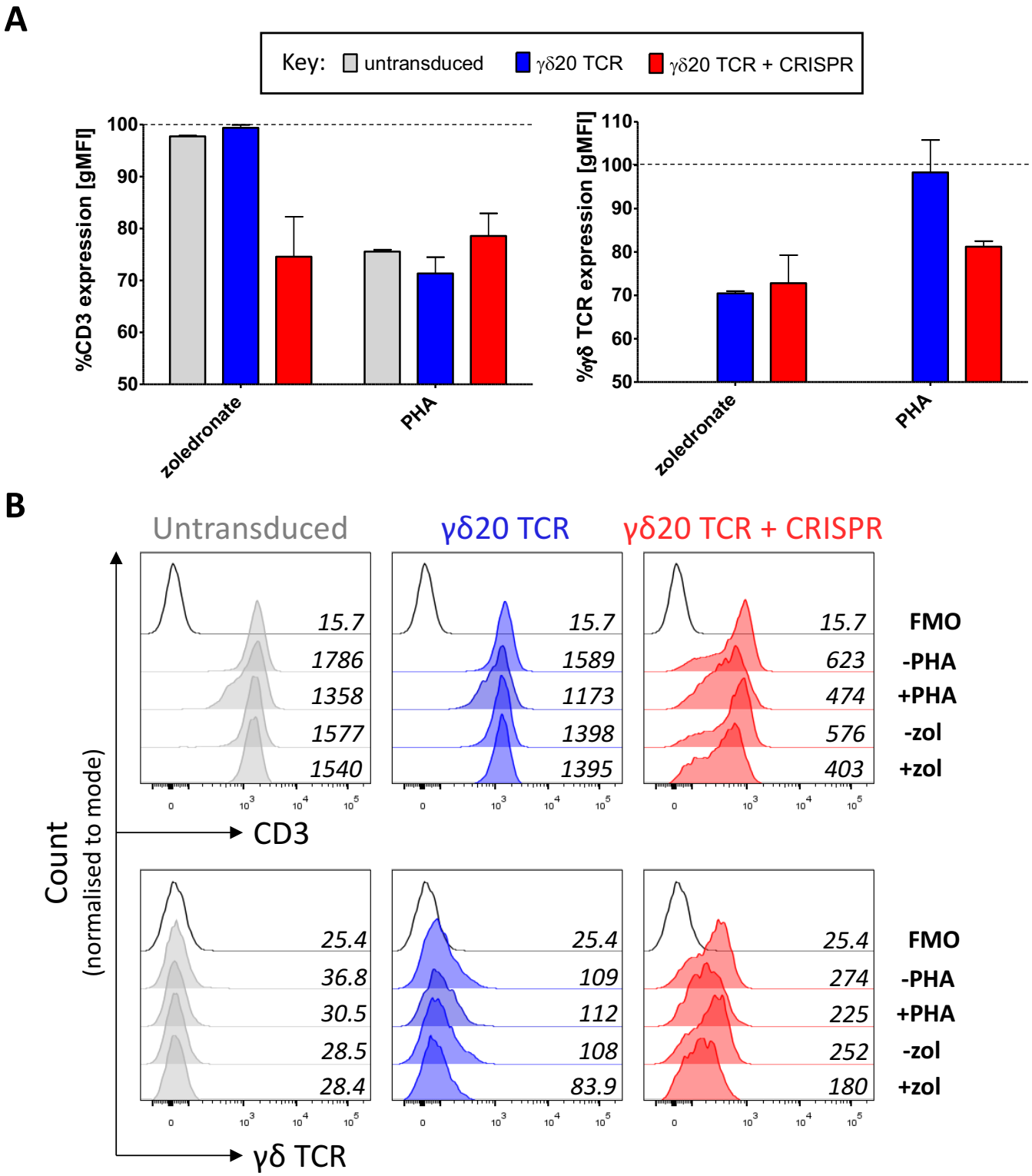
Supplementary Figure 1. Design and validation of gRNAs targeting *trbc* loci. (**A**) Expression of the endogenous αβ TCR-CD3 complex on T-cell leukemia line Jurkat E6.1, untransduced (wt) and transduced with 4 gRNAs targeting *trbc* gene segments (gRNA1-4). Numbers on dot plots indicate percentage of cells expressing αβ TCR-CD3 complex. (**B**) Alignment of tested gRNAs to *trbc1*, *trbc2* and codon-optimized (c.o.) *trbc* sequence used in transgenic αβ TCRs. Protospacer adjacent motif (PAM) is shown in red while blue highlight indicates nucleotide match between gRNA and *trbc*. gRNA sequence is shown as reverse complement in all four cases.



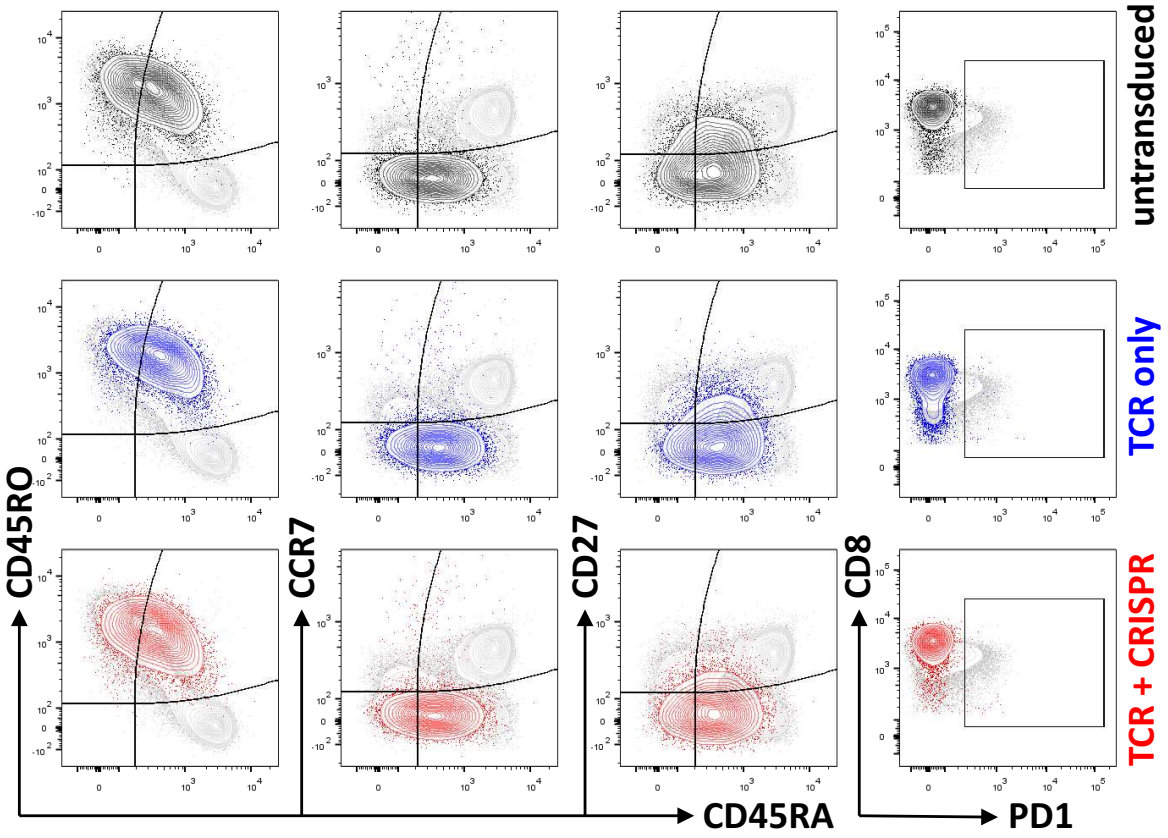
Supplementary Figure 2. The kinetics and efficacy of lentiviral transduction of primary T-cells. (**A**) T-cells (5×10^5 cells per condition) were isolated on day 0 and plated with CD3/CD28 beads. The cells were transduced with TCR only or TCR and CRISPR lentiviruses on day 1, and cultured until day 9, followed by magnetic pullout of $rCD2^+$ cells which were then plated with 2 µg/ml puromycin (TCR+CRISPR only). Puromycin selection was carried out until day 14 when the transduced cells (or a 5×10^5 untransduced cells) were expanded in presence of allogeneic irradiated feeders and PHA. The cells were counted every 2-4 days by trypan blue exclusion. (**B**) Following the initial selection, transduced or untransduced T-cells were expanded with allogeneic feeders and PHA every 14-28 days. The viable cells were counted after the expansion. (**C**) γδ20 transduced T-cells (with or without TCR-β CRISPR) were stained for rCD2 and αβ TCR on day 9 after isolation from peripheral blood (prior to any form of selection). Mean and standard deviation from three donors are shown.



Supplementary Figure 3. Functional response of TCR-transduced CD8⁺ T-cells shown as individual functions (IFNγ, CD107a, TNFα). The response of (**A**) γδ20 TCR-transduced CD8⁺ T-cells to LCL or LCL pre-incubated with zoledronate or (**B**) Mel13 TCR-transduced T-cells to a HLA-A2⁺ melanoma. Only viable CD3⁺ cells were included in the analysis while the gates for cells positive for a given function were set based on appropriate fluorescence minus one and biological controls. Numbers on dot plots refer to percentage of cells positive for a given function. Representative data from three donors and two independent experiments are shown.

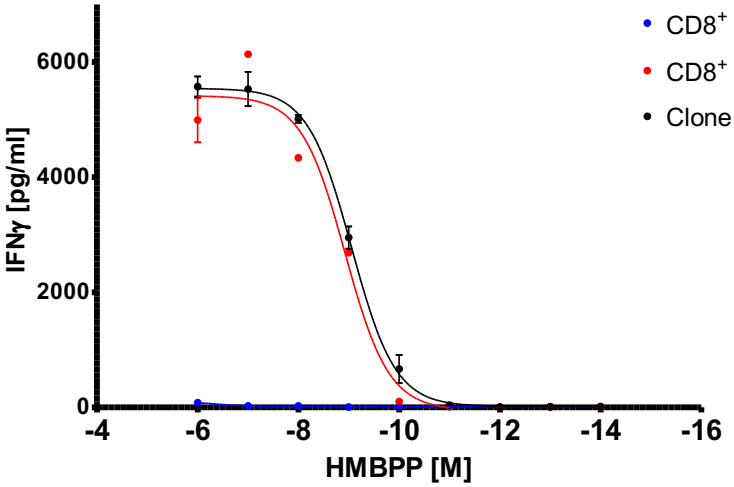


Supplementary Figure 4. TCR-CD3 complex undergoes downregulation upon antigen stimulation. γδ20 TCR transduced CD8⁺ T-cells and untransduced control cells were activated for 5 h with pan T-cell stimulus PHA or γδ20 TCR specific stimulus zoledronate (zol; in presence of an LCL cell line). Following incubation, cells were stained for CD3 and γδ TCR expression. Gating was performed to include only the viable T-cells. At least 10,000 viable events were acquired. (**A**) CD3 and TCR downregulation was calculated by dividing the geometric MFI of staining of stimulated by unstimulated sample, after subtracting FMO values. Mean and standard deviation is shown. (**B**) Representative staining for CD3 and γδ TCR. Numbers on histograms correspond to geometric MFI of staining. FMO, fluorescence minus one.



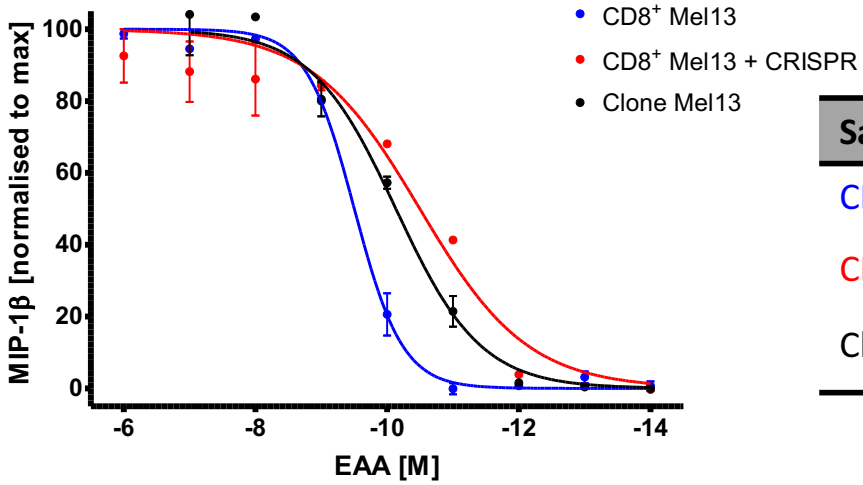
Supplementary Figure 5. Phenotypic profile of untransduced and transduced T-cells. Untransduced (top row), single transduced (TCR only; middle row) or double transduced (TCR+CRISPR; bottom row) T-cells were stained for phenotypic markers CD45RO, CD45RA, CCR7, CD27 and PD-1. Freshly isolated PBMC were stained in parallel as a control (shown in grey). Lymphocytes were gated based on scatter properties, followed by exclusion of doublets and dead cells. Only $CD3^{+}CD8^{+}$ cells were taken for further analysis.

A



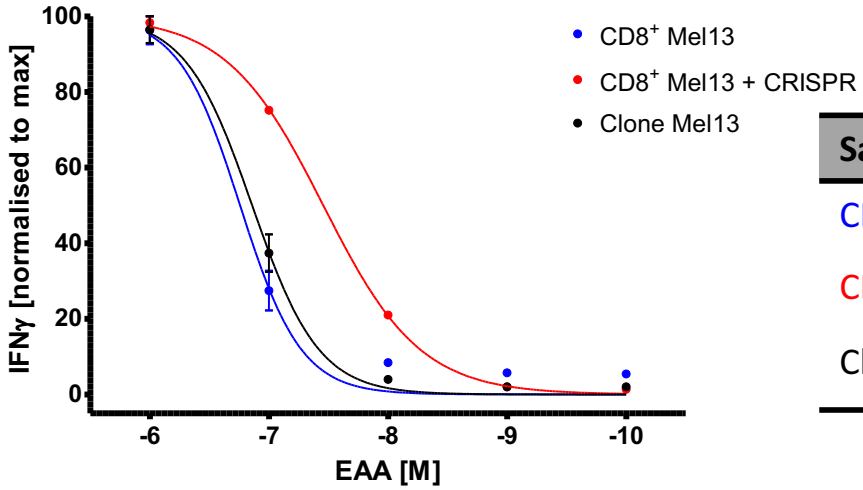
Sample	EC_{50} [M]
$CD8^+$ γδ20	ND
$CD8^+$ γδ20 + CRISPR	1.2×10^{-9}
Clone γδ20	8.8×10^{-10}

B



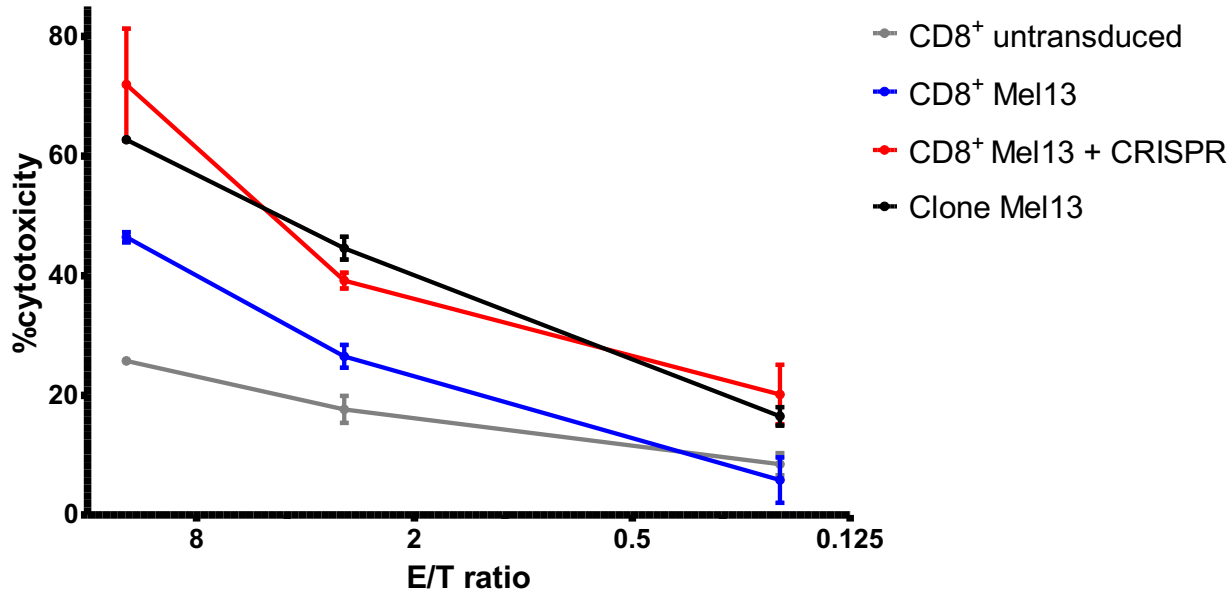
Sample	EC_{50} [M]
$CD8^+$ Mel13	3.1×10^{-10}
$CD8^+$ Mel13 + CRISPR	3.2×10^{-11}
Clone Mel13	7.3×10^{-10}

C

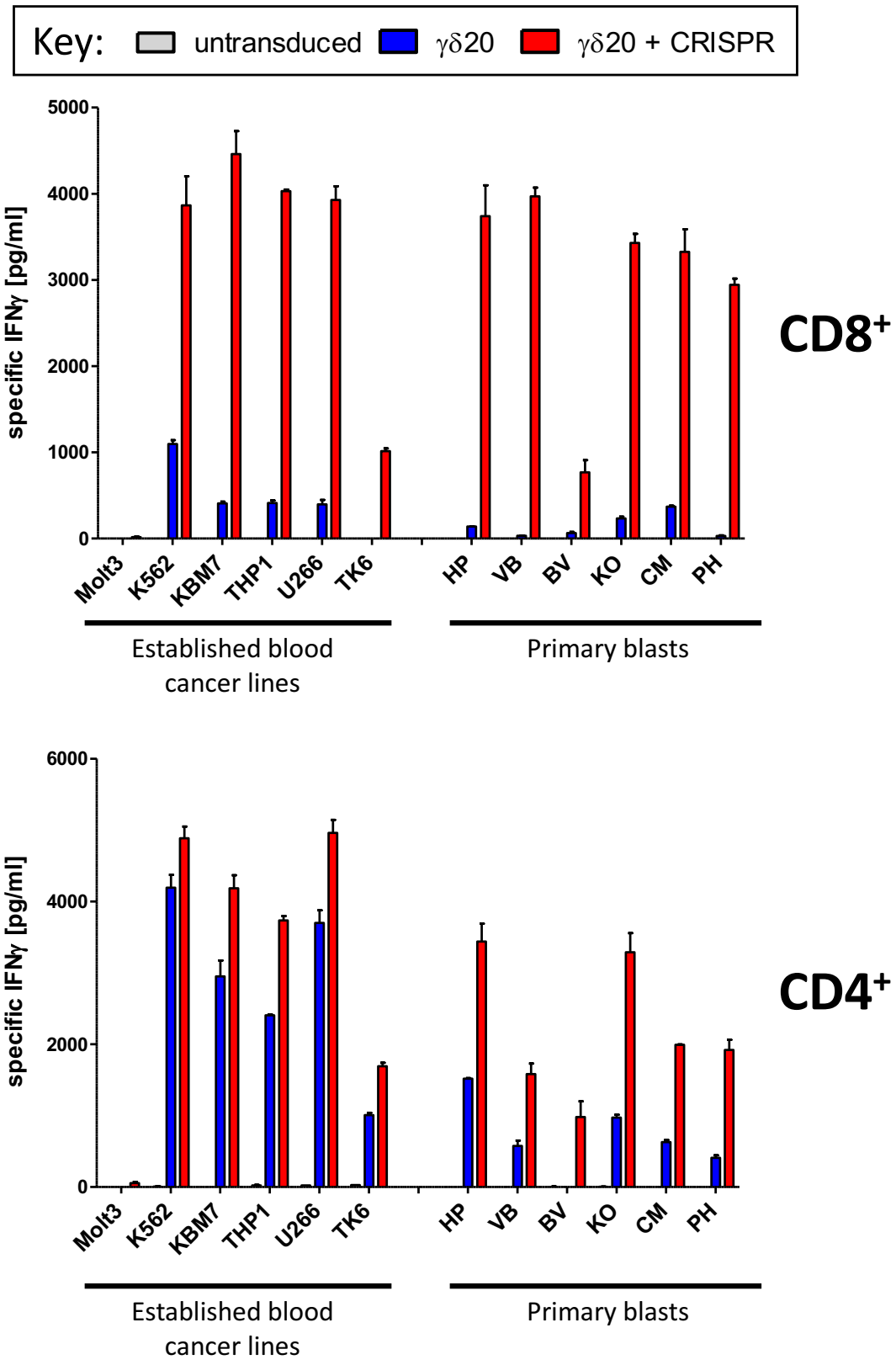


Sample	EC_{50} [M]
$CD8^+$ Mel13	1.7×10^{-7}
$CD8^+$ Mel13 + CRISPR	3.5×10^{-8}
Clone Mel13	1.4×10^{-7}

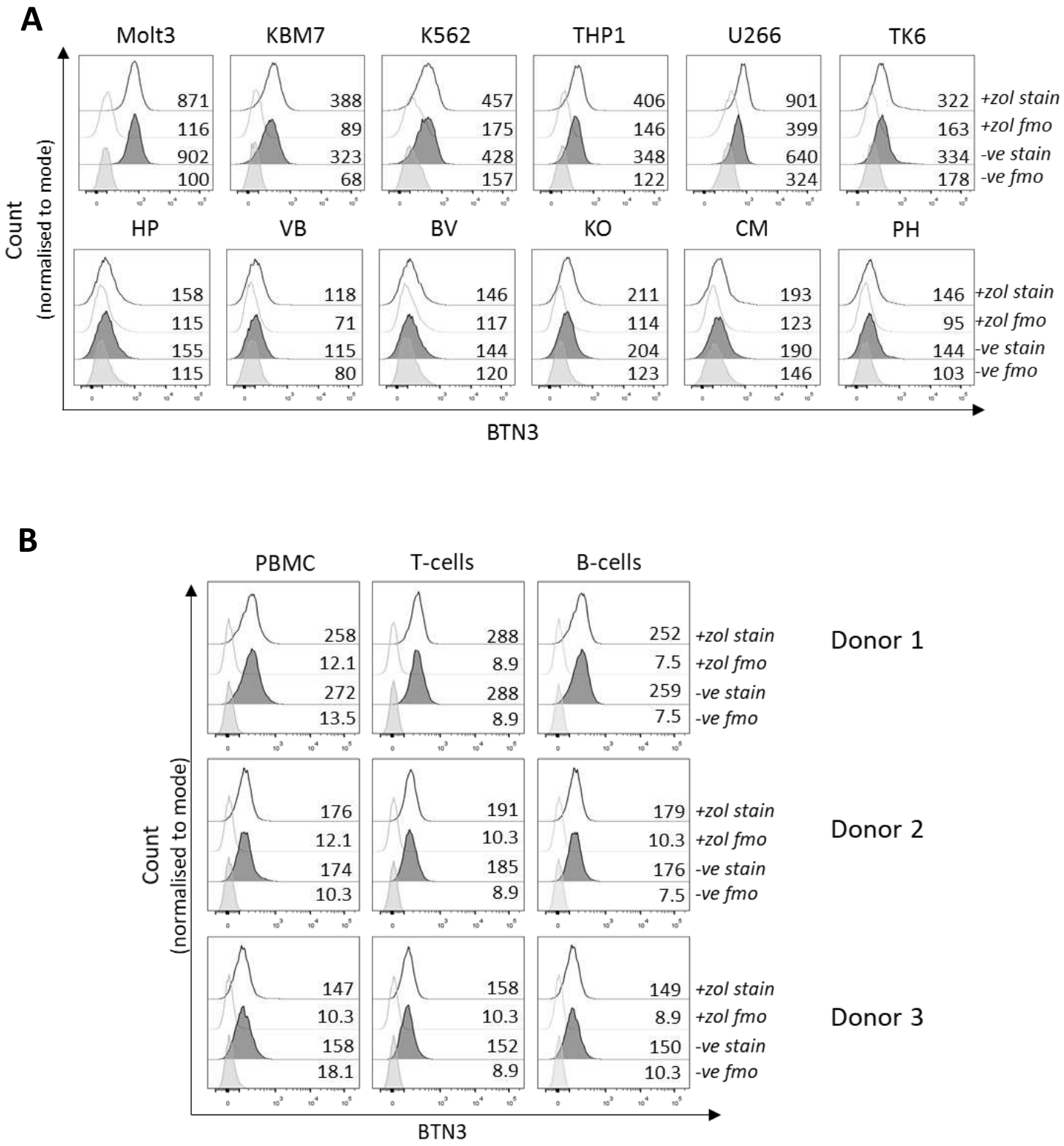
Supplementary Figure 6. Antigen sensitivity of γδ20 and Mel13 TCR transduced $CD8^+$ T-cells. (**A**) The sensitivity to the titrated antigen HMBPP was measured by IFNγ production after overnight incubation with the antigen and T2 cells used for antigen presentation. IFNγ concentration was normalized by subtracting the values of unstimulated cells. (**B**) The sensitivity to the titrated peptide EAAGIGILTV was measured by MIP-1β or (**C**) IFNγ production after overnight incubation with the antigen and T2 cells used for antigen presentation. The EC_{50} values were calculated in GraphPad Prism software by non-linear regression curve fitting.



Supplementary Figure 7. Mel13 TCR+CRISPR CD8+ T-cells show stronger cytotoxicity towards a melanoma cell line than Mel13 TCR-only transduced cells. 4 h cytotoxicity of transduced $CD8^+$ cells, as well as parental Mel13 T-cell clone, against a HLA-$A2^+$ melanoma cell line. Representative data are shown from two donors tested in two experiments carried out in duplicate.



Supplementary Figure 8. T-cells transduced with CRISPR replacement show a markedly stronger response to blood cancer lines than with standard transduction techniques. IFNγ secretion by transduced $CD8^+$ (top) or $CD4^+$ (bottom) T-cells after overnight co-incubation with a panel of established blood cancer lines of diverse lymphoid and myeloid origin, or patient-derived B ALL cells. Cancer cells were pre-incubated with zoledronate for 24 h before co-incubation with T-cells. IFNγ secretion was normalized by subtracting IFNγ produced by T-cells alone, and by cancer cells alone. No specific IFNγ secretion by T-cells was observed in absence of zoledronate pre-treatment. Representative data are shown from three donors and two experiments carried out in duplicate.



Supplementary Figure 9. Butyrophilin-3 expression on cancer cell lines and normal cells. (**A**) Cancer cell lines or (**B**) normal cells (untreated, -ve, or treated with 50 µM zoledronate, +zol) were stained with BTN3 antibody. The numbers on histograms refer to median fluorescence intensity of staining. Fmo, fluorescence minus one.