Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Addition of the C-terminus of CD6 to a chimeric antigen receptor enhances cytotoxicity and does not compromise expression

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doi:10.1111/imm.13009
Received 23 August 2018; revised 1 October 2018; accepted 2 October 2018.
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Summary

T cells expressing chimeric antigen receptors (CARs) are a promising new cancer immunotherapy that has now reached the clinic. CARs are synthetic receptors that redirect T cells towards a tumour-associated antigen and activate them through various fused signalling regions, for example derived from CD3ζ, 4-1BB or CD28. Analysis of the optimal combination of CAR components including signalling domains is not yet comprehensive and may vary with the particular application. The C-terminus of the T-cell surface receptor CD6 is critical for its co-stimulatory effects and signals through two phospho-tyrosine motifs that bind to the intracellular adaptor proteins GADS and SLP-76. Addition of the C terminus of CD6 did not compromise CAR expression, showing it was a stable moiety that can be used independently of the native receptor. A third-generation CAR containing 4-1BB, CD3ζ and the C terminus of CD6 (4-1BBz-CD6) enhanced interferon-γ release and cytotoxicity when compared with the second-generation 4-1BB CD3ζ (4-1BBz) CAR. The CD6 C terminus is a valuable addition to potential components for modular design of CARs to improve effector function, particularly cytotoxicity.

Keywords: CD6; chimeric antigen receptor; cytotoxicity; signal transduction; T cell.

Introduction

T cells expressing chimeric antigen receptors (CARs) are a novel cancer immunotherapy that shows great promise but still faces many challenges, especially with solid tumours.[1] The addition of a co-stimulatory cytoplasmic region to the principal signalling component, the CD3ζ chain, resulted in increased responses. Long-term survival and effector function of CAR T cells are preferentially enhanced by inclusion of the cytoplasmic regions of the tumour necrosis factor receptor family member 4-1BB or of CD28, respectively.[2,3] Combining cytoplasmic regions with different characteristics has indicated that increased signalling capability is beneficial when targeting solid tumours.[4] 'Third-generation' CARs containing both 4-1BB and CD28 in addition to CD3ζ cytoplasmic regions are now being tested in clinical trials.

The T-cell surface receptor CD6 can provide as strong a co-stimulatory signal to T cells as CD28.[5] Co-stimulation by CD6 is critically dependent on phosphorylation of two tyrosine residues at the C terminus of its long cytoplasmic region.[6,7] The specificity of these two tyrosine-based motifs, Y629 and Y662 for the adaptor proteins GADS and SLP-76, respectively, indicates that CD6 orchestrates a unique assembly of signalling proteins at the plasma membrane of T cells.[6,8] We tested a short region of the cytoplasmic tail of CD6 containing Y629 and Y662 for efficacy in enhancing CAR signalling. We show that addition of the C terminus of CD6 to a CAR containing 4-1BB and CD3ζ chain did not compromise expression and enhanced effector functions including cytotoxicity of primary human T cells.

Materials and methods

Constructs

The pFBneo vectors were constructed encoding CARs containing the extracellular and transmembrane regions of CD6 (GenBank: HSU34623, UniProt: P30203) fused to the

Abbreviations: 4-1BB CD3ζ (4-1BBz), 4-1BB CD3ζ and the C-terminus of CD6 (4-1BBz-CD6); CAR, chimeric antigen receptor; CD6 CD3ζ (CD6z), CD6 CD3ζ and the C-terminus of CD6 (CD6z-CD6)

complete cytoplasmic region of mouse CD3ζ chain (underlined) with the join FILLRAKFSR followed by a stop codon (CD6z) or an *Sal*I site at the C terminus to allow addition of a fragment encoding the C-terminal 47 amino acids (622–668) of human CD6 PPRSTSSGEWYQNFQPPPQPPSEEQFGCPGSPSPQPDSTDNDDYDDISAA (CD6z-CD6). Anti-CD19 CARs were constructed with the same CD6 C-terminal fragment and amino acids in the join to the C terminus of human CD3ζ chain. pCCL.EF1a – RQR8 containing the fmc63 anti-CD19 scFv, human CD8 stalk and transmembrane region, cytoplasmic regions of 4-1BB and CD3ζ chain[9] provided by Martin Pule (University College, London, UK) was re-engineered to delete the RQR8 suicide sequences and the CAR was transferred via 5′ *Bam*HI and 3′ *Xho*I sites into a pHR-SIN-BX-IRES-Em (Emerald) vector used previously.[6]

CAR cell production

Mouse T-cell hybridoma cells (2B4) were transduced with retroviral particles and cells expressing the human CD6 CAR protein were selected with a CD6 monoclonal antibody (mAb) (T12·1; ATCC, Manassas, VA) and Dynabeads anti-mouse IgG and grown in G418 (0·5–1 mg/ml).[7] Human $CD4^+$ and $CD8^+$ T cells were isolated from blood cones from anonymous donors using RosetteSep isolation kits (Stemcell Technologies, Cambridge, UK). T cells were activated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher Scientific, Hemel Hempstead, UK) in the presence of interleukin-2 (IL-2) (100 U/ml). Activated T cells were transduced with lentiviral particles containing the CAR constructs and CAR-expressing cells were further enriched with Dynabeads M-280 Streptavidin and Fab-specific anti-mouse IgG biotin (Sigma-Aldrich, Co. Ltd, Cambridge, UK). Expression of CARs was detected by flow cytometry using the same antibodies as for selection and Streptavidin Dylight 650 (Thermo Fisher Scientific, Hemel Hempstead, UK) on a FACSCalibur machine (BD Biosciences, Wokingham, UK).

Cytokine secretion

Mouse T-cell hydridoma cells (10^5) and $CD4^+$ CAR T cells (10^5) were stimulated with plate-bound CD6 mAb (T12.1) or varying numbers of $CD19^+$ Daudi B-cell lymphoma cells (0×10^5 to 4×10^5), respectively for 18 hr at 37°. Supernatants were then analysed for the presence of the cytokines IL-2 and interferon γ (IFN-γ) with mouse or human (Peprotech, EC Ltd, London, UK) IL-2 and IFN-γ enzyme linked immunoabsorbent assay (ELISA) reagents or kits.

Cytotoxic granule secretion

The $CD8^+$ CAR T cells (5×10^4) were stimulated with varying numbers of $CD19^+$ Daudi cells in the presence of Monensin (2 μM; BioLegend) and anti-human CD107a-allophycocyanin (1 : 50; Miltenyi Biotec) for 5 hr at 37°. The percentage of CD107a-positive T cells was then analysed by flow cytometry for the allophycocyanin signal as an indication of cytotoxic granule secretion and for each experiment, data were normalized to the mean value for 4-1BBz cells cultured with 4×10^5 Daudi cells.

Cytotoxicity assayed by flow cytometry and lactate dehydrogenase release

$CD19^+$ Daudi cells ($0{\cdot}4 \times 10^4$) were loaded with carboxyfluorescein succinimidyl ester (CFSE, 10 μM; Thermo Fisher Scientific), and CD19-Jurkat cells ($0{\cdot}4 \times 10^4$) were loaded with CFSE (1 μM), and the labelled cell lines were mixed in a 1 : 1 ratio. $CD8^+$ T cells were labelled with Celltrace Far Red (5 μM) to distinguish the transduced $EGFP^+$ T cells from CFSE-labelled target cells. Labelled $CD8^+$ T cells were added to the target cells at varying effector: target ratios and incubated for 16 hr at 37°. The ratio of Daudi cells to Jurkat cells was then determined by flow cytometry as an indication of specific killing of $CD19^+$ Daudi cells. Additionally, killing of $CD19^+$ (Daudi) cells ($0{\cdot}4 \times 10^5$) at various effector : target ratios was measured by release of lactate dehydrogenase using a Cytotox 96 kit (Promega UK Ltd, Southampton, UK).

Data analysis

In each assay, replicates from all experiments were analysed using an *F*-test or paired *t*-test in GRAPHPAD PRISM.

Results

A CAR containing the C terminus of CD6 is expressed and enhances T-cell activation

We first tested the hypothesis that a C-terminal stretch of the CD6 cytoplasmic region containing the Y629 and Y662 tyrosine-based motifs would enhance the function of a CAR in a mouse T-cell hybridoma model.[10] We have previously observed signalling through this region of full-length human CD6 in these cells.[7] As this region in native CD6 is distal to the membrane, the C-terminus of CD6 was fused to the C-terminus of a chimeric receptor containing the extracellular and transmembrane regions of human CD6 and the cytoplasmic region of mouse CD3ζ chain. The second-generation CD6 containing CAR (CD6z-CD6) was expressed as well as the first-generation CAR (CD6z) in a hybridoma cell line (Fig. 1a). In response to stimulation with immobilized CD6 mAb, cells expressing the CAR containing the C terminus of CD6 produced two-to three-fold more IL-2 in 18 hr compared with the first-generation CAR (Fig. 1b). These data showed that addition of a short region from the C

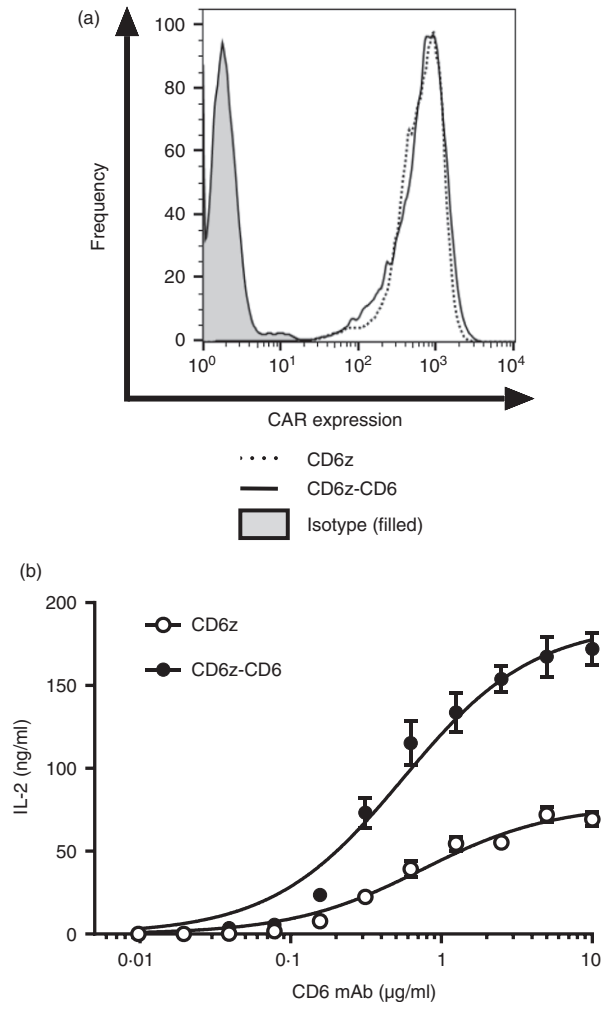

Figure 1. A chimeric antigen receptor (CAR) containing the C terminus of CD6 is expressed and enhances T-cell activation. (a) Flow cytometric analysis of CD6 CAR expression on mouse T-cell hybridoma cells with a CD6 mAb. (b) Secreted IL-2 from 10^5 hybridoma cells stimulated with the indicated concentrations of CD6 mAb for 18 hr. Experiments were conducted three times in triplicate. Means ± SEM are shown. Curves for CD6z and CD6z-CD6 cells were different $P < 0{\cdot}0001$.

terminus of CD6 to a CAR did not compromise expression and that the C terminus of CD6 isolated from its native receptor enhanced T-cell activation.

The CD6 C terminus does not affect expression of a CAR in primary human T cells

We proceeded to test the C terminus of CD6 in an anti-CD19 CAR. We constructed a third-generation anti-CD19 CAR containing the cytoplasmic regions of 4-1BB, CD3ζ and the C terminus of CD6 (4-1BBz-CD6). Primary $CD4^+$ and $CD8^+$ T cells were transduced with the second generation, anti-CD19 4-1BBz, or third-generation, anti-CD19 4-1BBz-CD6 CARs and analysed for expression by flow cytometry. Anti-CD19 4-1BBz and anti-CD19 4-1BBz-CD6 were expressed at the same level on $CD4^+$ and $CD8^+$ T cells (Fig. 2a). Addition of the C terminus of CD6 to the distal end of a second-generation CAR did not compromise expression levels.

Interferon-γ release from primary T cells is increased by addition of the C terminus of CD6 to a CAR

As a first assessment of the potential of the C terminus to enhance CAR signalling in primary T cells, we measured cytokine release by transduced $CD4^+$ T cells. Stimulation of $CD4^+$ CAR T cells with $CD19^+$ (Daudi) cells resulted in IL-2 and IFN-γ production. (Fig. 2b,c). Addition of the C terminus of CD6 to the CAR did not further increase IL-2 production by the primary T cells (Fig. 2b), but it did increase release of IFN-γ (Fig. 2c). These data showed that the C terminus of CD6 can mediate signal transduction in the context of a CAR in human T cells.

Cytotoxic granule release from primary T cells is increased by addition of the C terminus of CD6 to a CAR

The preferential effect of CD6 signalling on IFN-γ compared with IL-2 release from $CD4^+$ T cells suggested that the CD6 moiety might be more relevant for enhancing effector function than for proliferation in response to autocrine IL-2. A key effector function of CAR T cells is cytotoxicity. $CD8^+$ CAR T cells in the tumour environment need to release cytotoxic granules to kill tumour cells. A common assay to measure the release of cytotoxic granules is to stain for CD107a, a lysosomal marker that appears on the cell surface after degranulation. Stimulating transduced $CD8^+$ CAR T cells with $CD19^+$ target cells led to an increase of CD107a staining with the 4-1BBz-CD6 compared with the 4-1BBz CAR (Fig. 3a). The addition of the C terminus of CD6 to a CAR enhanced $CD8^+$ T-cell degranulation, indicating more effective killing.

Tumour cell killing by primary T cells is increased by addition of the C terminus of CD6 to a CAR

To test more directly the effect of the C terminus of CD6 in the 4-1BBz CAR on tumour cell killing, cytotoxicity assays were conducted (Fig. 3b,c). $CD19^+$ target (Daudi) cells and $CD19^-$ (Jurkat) cells were labelled with different concentrations of the cell dye, CFSE and incubated with $CD8^+$ CAR T cells. The ratio of $CD19^+$ to $CD19^-$ cells was then analysed by flow cytometry. The third-generation, 4-1BBz-CD6 CAR T cells achieved significantly higher killing of $CD19^+$ cells than the second-generation, 4-1BBz CAR T cells (Fig. 3b). Enhanced killing in the presence of the C terminus of CD6 was confirmed with

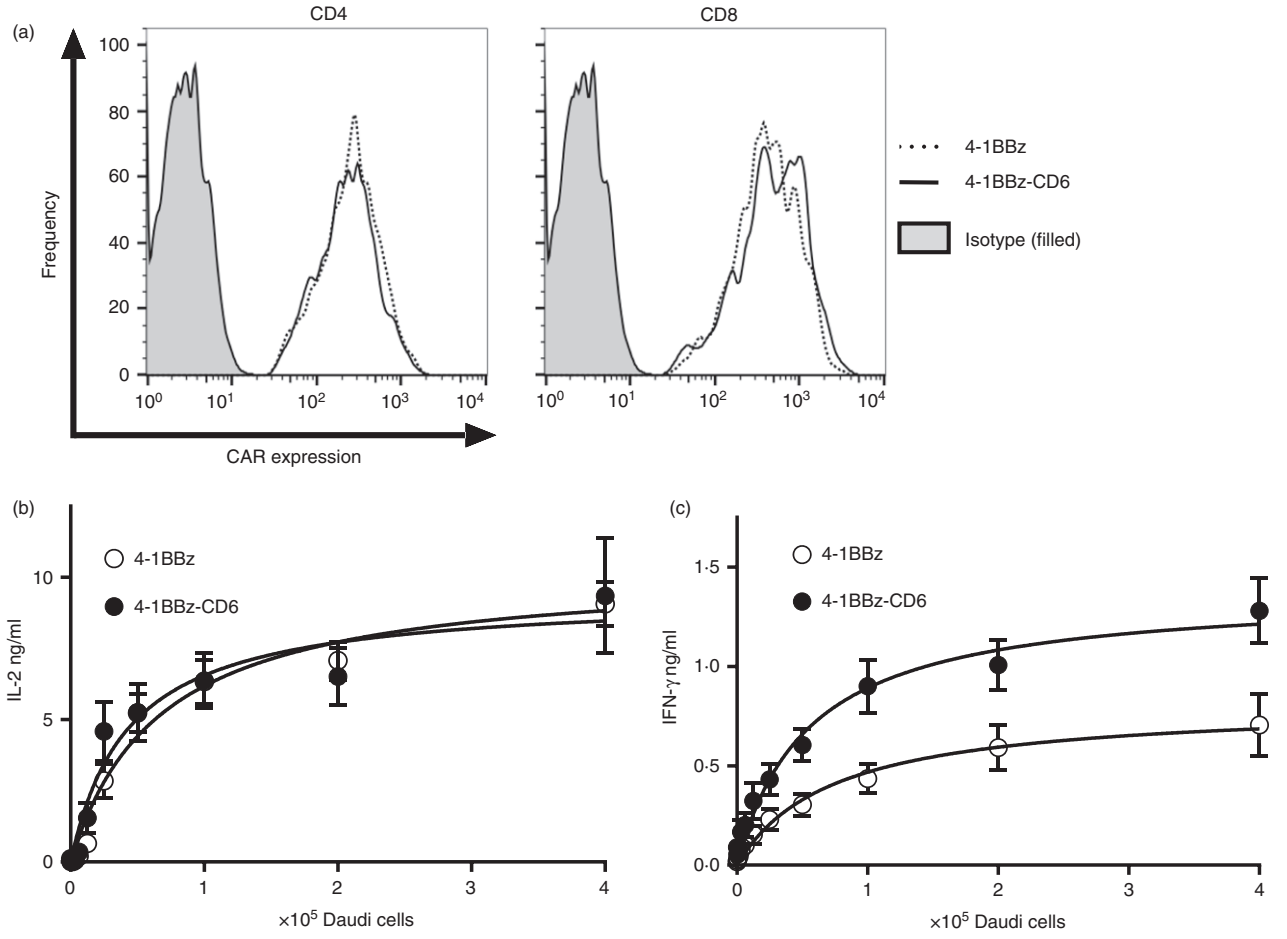

Figure 2. A chimeric antigen receptor (CAR) containing the C terminus of CD6 is expressed and enhances IFN-γ release. (a) Flow cytometric analysis of anti-CD19 CAR expression on $CD4^+$ and $CD8^+$ T cells with a Fab-specific anti-mouse IgG antibody. (b, c) Secreted interleukin-2 (IL-2) (b) and interferon- γ (IFN-γ) (c) from 10^5 $CD4^+$ CAR T cells stimulated with the indicated numbers of $CD19^+$ Daudi cells for 18 h. Experiments were conducted twice in duplicate for each donor ($n \geq 3$ except untransduced, $n = 1$). Means ± SEM are shown. Compared with 4-1BBz cells, IL-2 and IFN-γ production by untransduced cells with 4×10^5 Daudi cells was <20% (not shown). Curves for IFN-γ production by 4-1BBz and 4-1BBz-CD6 cells were different $P < 0{\cdot}0001$.

an additional killing assay that assesses the release of lactate dehydrogenase during cell death (Fig. 3c).

Discussion

Assembly of multiple components of the cytoplasmic region of CARs is aimed at enhancing both effector function and *in vivo* persistence of the transferred T cells. Cytoplasmic regions containing tyrosine-based motifs such as CD28, ICOS and CD244 have been shown to contribute to both these functions.[2,4,11] Comparison between CD28- and 4-1BB-containing CARs indicated the former cytoplasmic region is superior in enhancing effector function and the latter is important for persistence of the CAR T cells.[3] Based on the *in vitro* data, inclusion of CD6 is more relevant for enhancement of IFN-γ production and killing of $CD19^+$ cells and complementing 4-1BB cytoplasmic-region-dependent persistence. The mechanism of action of the isolated C-terminal region of CD6 is likely to involve the same interactions as in the native receptor.[6–8] Phosphorylation-dependent interactions of the two tyrosine motifs seem to be the dominant interactions mediated by this region of the CD6 cytoplasmic region. Attempts to identify interactions of the proline-rich region in between Y629 and Y662 using a peptide encompassing amino acids 632–656 did not yield a specific binding partner.[7]

Proliferation of CAR T cells is critical for clinical efficacy.[2] *In vitro* analysis of the effects of the CD6 fragment on the anti-CD19 CAR function indicated that it would not play a significant role in promoting survival through production of IL-2. The C terminus of CD6 does have potential to enhance IL-2 production as observed in our studies of signalling by CD6[6,7] and in a chimeric antigen receptor form in hybridoma cells. Whether this is sufficient to maintain adequate proliferation of CAR T cells

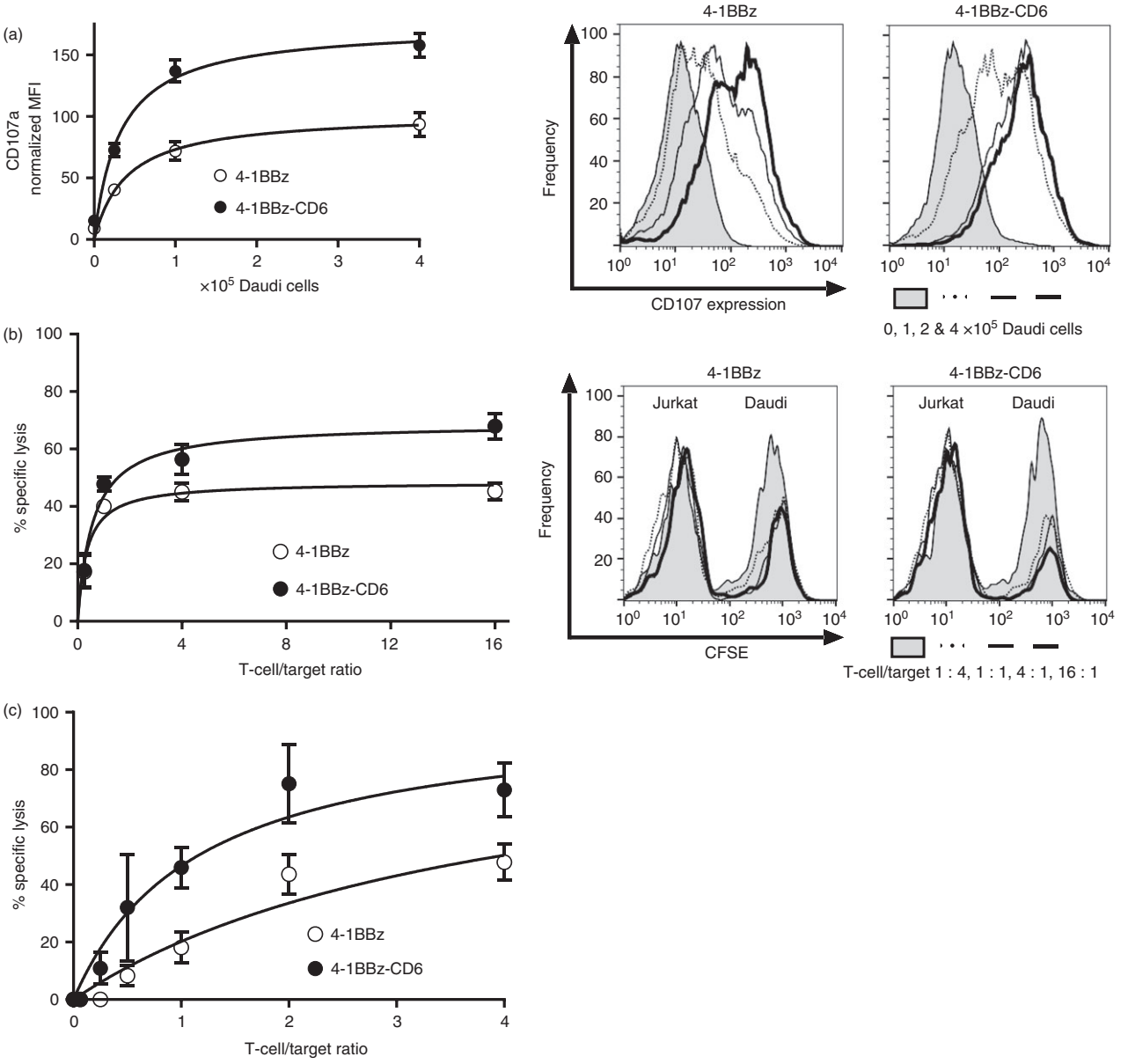

Figure 3. Addition of the C terminus of CD6 to a chimeric antigen receptor (CAR) enhanced cytotoxicity. (a) $CD8^+$ T cells stimulated with the indicated numbers of Daudi cells and anti-CD107a-APC were analysed by flow cytometry (a representative example is shown in the righthand panel) and for each experiment, data were normalized to the mean value for 4-1BB ζ cells cultured with 4×10^5 Daudi cells. (b) $CD19^+$ (Daudi) and $CD19^-$ (Jurkat) cells labelled with CFSE were incubated with $CD8^+$ CAR T cells at the indicated ratios and specific killing of $CD19^+$ Daudi cells was analysed by flow cytometry (a representative example is shown in righthand panel). (c) Killing of $CD19^+$ (Daudi) cells at the indicated T-cell : target ratios was measured by release of lactate dehydrogenase. All experiments were conducted twice in duplicate for each donor ($n = 3$). Means ± SEM are shown. In (a) and (c), curves for CD107a expression ($P < 0{\cdot}0001$) and release of lactate dehydrogenase ($P < 0{\cdot}05$) by 4-1BBz and 4-1BBz-CD6 cells were different and in (b) compared with 4-1BBz, 4-1BBz-CD6 cells enhanced killing at T-cell : target ratios 4 : 1 ($P < 0{\cdot}05$) and 16 : 1 ($P < 0{\cdot}001$).

in vivo remains to be tested. Preliminary experiments indicated that the C terminus of CD6 was less effective compared with the CD28 cytoplasmic region in promoting proliferation (unpublished results).

One key asset of the C terminus of CD6 is that it is a stable moiety. The positioning of the C-terminal CD6 fragment in the CAR was based on its position in native CD6. The CD6 fragment was functional in isolation from the native receptor. It was also well expressed in a membrane proximal position (Philip Kruger, personal communication) which may work equally well. The adaptor protein recruited by the C terminus of CD6, SLP-76 was functional when placed in a membrane proximal position in a chimeric receptor.[12]

Addition of the C terminus of CD6 to the distal end of first- and second-generation CARs did not compromise expression unlike the CD28 domain.[13] In preclinical trials, the addition of CD28 and ICOS cytoplasmic regions, both from the same family of receptors, reduced expression of CARs and was sensitive to positioning and not effectively expressed distal to the membrane.[4,9] Reduced CAR expression on addition of increasing numbers of cytoplasmic components may be indicative of a less stable protein. Lower expression limits efficacy and complicates comparisons between CARs.[4,9] An effective stable cytoplasmic region which does not compromise expression while enhancing function is valuable for further development and optimization of CARs and with reference to a recent report on exploiting CD6 as a homing system receptor, is also potentially relevant to their delivery.[14]

Acknowledgements

JB was supported by an EIT Health Fellowship, the CIU Trust and BP by the European Union's Seventh Framework Programme, 'ATECT', under grant agreement no 602239. We thank Martin Pule and Omer Dushek and Jesús Siller Farfán for helpful discussion and Philipp Kruger and Benjamin Salzer, who also read the manuscript.

Disclosures

JB and MHB are named as 'inventors' on the Patent Cooperation Treaty application on the use of the C terminus of CD6 in a CAR format, which has now been published (as WO2018/025052(A1)).

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