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A novel simplified method of generating cytomegalovirus-specific cytokine-induced killer cells of high specificity and superior potency with GMP compliance

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ABSTRACT

We describe a method of rendering polyclonal cytokine-induced killer cells (CIK) specific against cytomegalovirus (CMV), focusing on GMP compliance. Peripheral blood mononuclear cells (PBMNC) are stimulated with pooled CMV peptides pp65 and IE-1 for 16–24 h and the reactive T cell subset which up-regulate CD137 is further co-stimulated with anti-CD137, followed by expansion in G-Rex flasks under standard CIK culture condition. This method generates a large number CMV-specific CIK with superior potency compared to published method currently in clinical trials. The cytotoxicity as measured by chromium release assay correlates with the upregulation of CD107a upon peptide re-challenge as measured by flow cytometry. CMV-CIK at maturity consist of mainly late effector memory CD8 T cells and have a skewed TCR repertoire with preferential expansion of a few families. Such CMV-CIK retain their function after freezing and thawing. CMV-CIK thus generated is ready for clinical trial against drug-resistant CMV disease.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative treatment for patients with hematological malignancies but is fraught with risk of opportunistic infections. Reactivation of Cytomegalovirus (CMV) or other dormant viruses following allo-HSCT are serious complications in these patients. While anti-viral drugs are effective for some viruses, drug toxicity and resistance are two major problems. Adoptive transfer of virus-specific T cells after allo-HSCT to restore virus specific cellular immunity and suppress viremia has emerged as an alternative treatment [1]. Various methods have been developed to isolate and expand virus-specific T cells for clinical use. These include direct isolation of virus-specific T cells from leukapheresis product [2–5], or expansion with antigen presenting cells (APC) either engineered to express CMV phosphoprotein 65 (pp65) [6,7] or pulsed with pool of overlapping CMV peptides [8,9], and have demonstrated efficacy in clinical trials.

Cytokine-induced killer cells (CIK) are immune effectors consisting of CD3 + CD56 + NK-like T cells, CD3-CD56 + NK cells and CD3 + CD56- T cells [10,11]. CIK are polyclonal T cells that exhibit non-major histocompatibility complex (MHC) restricted cytotoxicity [12], generated by culturing peripheral blood mononuclear cells (PBMNC) in the presence of interferon-gamma (IFN-γ), monoclonal antibodies against CD3 (OKT3) and interleukin-2 (IL-2) [10,11]. CIK have been used in clinical trials for both hematological malignancies [13–17] and solid tumours [17,18].

CD137 (4–1BB) is an antigen belonging to the tumour necrosis factor receptor family, expressed on the surface of activated T cells, NK cells and dendritic cells [19]. The ligation of CD137 mediates co-stimulating effect, thereby promoting T cell proliferation and cytokine production [20]. Humanized antibodies against 4–1BB are currently in several clinical trials against various cancers [19].

Here we describe a robust and simple system that can generate CMV-specific CIK (CMV-CIK) multiple folds within 2–3 weeks. We show

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; CMV, Cytomegalovirus; APC, antigen presenting cells; pp65, phosphoprotein 65; CIK, Cytokine-induced killer cells; MHC, major histocompatibility complex; PBMNC, peripheral blood mononuclear cells; CMV-CIK, Cytomegalovirus-specific Cytokine-induced killer cells; CMV-CTL, Cytomegalovirus -specific cytotoxic T lymphocytes; CMV-R-CIK, Cytomegalovirus -reactive Cytokine-induced killer cells; CMV-NR-CIK, Cytomegalovirus - non-reactive Cytokine-induced killer cells; CMV-US-CIK, Cytomegalovirus -unsorted- Cytokine-induced killer cells; TCR Vβ, T cell receptor V beta

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that through CMV peptide-induced CD137 up-regulation and co-stimulation, large number of CMV-CIK of high potency and specificity can be generated from frozen PBMNC. We further show that this method generates T cells of greater anti-CMV activity than the published method that generates CMV-specific cytotoxic T lymphocytes (CMV-CTL) by pooled peptide stimulation and expansion with IL4 and IL7, currently used in clinical trials [8,21].

2. Materials and methods

2.1. Cells for culture

PBMNC was harvested by ficoll gradient centrifugation of discarded whole blood obtained from patients with iron overload or polycythemia undergoing therapeutic venesection, with approval from Institutional Review Board and informed consent from patients. These were cryopreserved in aliquots in 10% DMSO/10% FBS (Bovogen Biologicals Pty Ltd., East Keilor, Vic, Australia)/RPMI (HyClone Laboratories, Inc., South Logan, Utah) and stored in liquid nitrogen.

Subsequent experiments comparing method of generating anti CMV effectors also utilized MNC isolated from leukocyte cones which are discarded by-products of plateletpheresis from healthy platelet donors.

2.2. Screening for presence of CMV reactivity

PBMNC was thawed and incubated at 1.5 million cells in 150uL per well in 96 well plates without and with CMV pp65 and IE-1 pooled peptides (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany or JPT Peptide Technologies GmbH, Berlin, Germany) according to manufacturers' instruction for 18–24 h. Cells were harvested and stained with CD3 PC5 (Biolegend, San Deigo, CA; Clone UCHT1), CD4 FITC (BD Biosciences Pharmingen, San Deigo, CA, USA; Clone RPA-T4), CD137 PE (Miltenyi Biotec, Bergisch Gladbach, Germany; clone 4B4–1), and analysed by flow cytometry (Cytomics FC500 Flow Cytometer, Beckman Coulter). Samples with CD137+ cells comprising of at least 0.5% of total CD3 cells were deemed to have adequate CMV reactive T cells and selected for culture of CMV-CIK.

2.3. Generation of CMV-CIK

The same method for screening applied on a larger scale was used to start culture. Cells stimulated for 18-24 h with CMV pooled peptides were harvested and stained with anti-CD137-PE followed by anti PE microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and sorted through magnetic column (Miltenyi, LS) into CD137-positive and CD137 -negative fractions. In some cultures, a fraction of the CD137 labelled cells was put aside without being subjected to column sorting. These 3 fractions, designated CMV-reactive CIK (CMV-R-CIK), CMV-non-reactive CIK (CMV-NR-CIK) and CMV-unsorted-CIK (CMV-US-CIK) respectively, were then started on culture in T25 or G-Rex 10 cell culture flask (Wilson Wolf, New Brighton, MN) under standard cytokine conditions for CIK cultures. Briefly, this involved addition of IFN-γ (Imukin®, Boehringer Ingelheim RCV GmbH & Co KG, Vienna, Austria) at 1000 U/ml on D1, OKT3 (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) at 50 ng/ml and IL-2 (Proleukin®, Novartis Pharmaceuticals UK Ltd., Horsham, West Sussex) at 300 U/ml on D2. Culture medium comprising of 10% FBS/RPMI was added twice a week and IL-2 was replenished weekly.

2.4. Generation of CMV-CTL by published method

In 3 additional experiments, CMV-specific T cells were generated based on published method [8,21]. MNC isolated from cones were loaded with CMV pooled peptides and expanded in IL4 at 800u/ml (Human IL-4, premium grade. Miltenyi) and IL-7 at 20 ng/ml (Human Recombinant IL-7, Stemcell Technologies). T cells generated by this method are designated CMV-CTL to distinguish from CMV-US-CIK, and both were cultured in parallel in G-Rex flasks. In order to achieve xeno-free culture condition for these cultures, an optimized medium comprising of 10% AB serum in Immunocult-XF (Stem cell technologies) was chosen after comparing a few xeno-free media against the standard 10%FCS/RPMI. Cytokines were replenished weekly.

2.5. Assay for CMV specificity

Effectors (CIK and CTL) at between D14 to D21 were harvested to assay for reactivity against CMV. The standard 4-hour chromium release assay was employed to study the cytotoxicity and specificity against CMV. Targets included PHA blasts generated from thawed autologous PBMNC cultured with PHA (Sigma Aldrich, St. Louis, MO) at 2.5 μg/ml and IL-2 at 100 U/ml. PHA blasts were used without and with loading of CMV pooled peptides, labelled with 150 uCi radioactive chromium (^{51}Cr, Perkin Elmer) for 3 h, washed and plated in triplicate in 96-well round-bottom plate. Effectors were added to the targets at various effector: target (E: T) ratios. Minimal and maximal release wells were set up in the same plate, with white cell lysis buffer added to the maximal release wells. Radioactivity of the supernatant was counted by a gamma reader (Wallac Wizard 1470 Gamma counter) after 4 h of incubation.

Expression of CD107a as a degranulation marker was assayed by incubating effectors with and without pooled CMV peptides together with CD107a PC7 (Biolegend Inc., San Diego, CA; Clone H4A3) for 6 h. The proportion of CD107a positive cells which represents proportion of effectors reactive to CMV pooled peptides was measured by flow cytometry, with those incubated without peptides serving as control.

2.6. Phenotypic characterization

Pre-culture PBMNC and effectors were stained for CD3FITC (clone UCHT1), CD56PE (B159), CD4 FITC (Clone RPA-T4), CD8PE (HIT8a), CD45RO (Clone UCHL1), CD62L (Clone DREG-56), CD27 (Clone M-T271) and CD28 (Clone CD28.2, all from BD Biosciences Pharmingen) and analysed for composition of CD3 + CD56+ NK-like T cells, CD4/CD8 subsets and memory T cell subsets. Furthermore, T cell receptor V beta (TCR Vβ) repertoire of pre-culture PBMNC and CMV-R-CIK was also studied by flow cytometry (IO test®; Immunotech, SAS, Beckman Coulter, Marseille cedex, France).

2.7. Statistical analysis

Comparison of fold expansion and % cytotoxicity between CMV-R-CIK and CMV-NR-CIK was done using Wilcoxon signed rank test for paired samples, and statistical significance was taken as p value of < 0.05.

3. Results

3.1. Ex-vivo generation of CMV-CIK

PBMNC from 7 venesected blood samples were used for generation of CMV-CIK, see Table 1. These samples had been screened and had shown presence of CMV reactivity as reflected by the expression of CD137 in a small subset after overnight incubation with pooled CMV peptides, at a median of 1.27% (range 0.72% to 5.46%). After labelling with anti-CD137PE followed by anti-PE microbeads and sorted through magnetic column, this population was enriched to a median of 46.34% (range 3.05% to 92.35%) but was reduced to a low cell number. From a median of 72.4 million thawed PBMNC (range 42 to 96 million) which underwent overnight stimulation and column sorting, 0.22–4.41 million CD137 positive cells (median 0.35 million) were recovered for CMV-R-CIK culture (Table 1), together with 6–40.9 million CD137 negative cells (median 10.9 million) for CMV-NR-CIK culture. Despite

Table 1
Pre- and post- expansion characterization of the 7 cultures

ID	Reason for venesection	%CD137+ cells in CD3+ cells		Cell count (million)			% Recovery of post thaw cell count		D14 culture	D14 fold expansion	
		Post- stimulation (%)	Post-sort (%)	Post-thaw (a)	Post- overnight stimulation (b)	Post-sort CD137+ fraction (c)	Post- stimulation (b/a)	Post-sort (c/a)	Count (million) (d)	Over post- stimulation (d/b)	Over post- sort (d/c)
UPN 1	Iron overload post allo-HSCT	5.46	ND	55	28	0.5	50.00%	0.91%	20.50	0.75	41.00
UPN 2	Iron overload post allo-HSCT	5.04	92.35	75	38	4.41	50.67%	5.88%	97.60	2.57	22.13
UPN 3	Iron overload post allo-HSCT	1.45	73.04	42	18	0.3	42.38%	0.71%	63.04	3.54	210.13
UPN 4	Secondary polycythemia	0.72	3.05	75	30	0.85	40.00%	1.13%	111.60	3.72	131.29
UPN 5	Secondary polycythemia	1.27	66.23	60	43	0.22	72.27%	0.37%	136.00	3.16	618.18
UPN 6	Polycythemia rubra vera	1.23	26.45	72	34	0.33	46.63%	0.46%	18.60	0.55	56.36
UPN 7	Secondary polycythemia	1.13	20.38	96	87	0.35	91.04%	0.36%	219.76	2.51	627.88
Median		1.27	46.34	72.38	33.75	0.35	0.5	0.71%	97.60	2.57	131.29

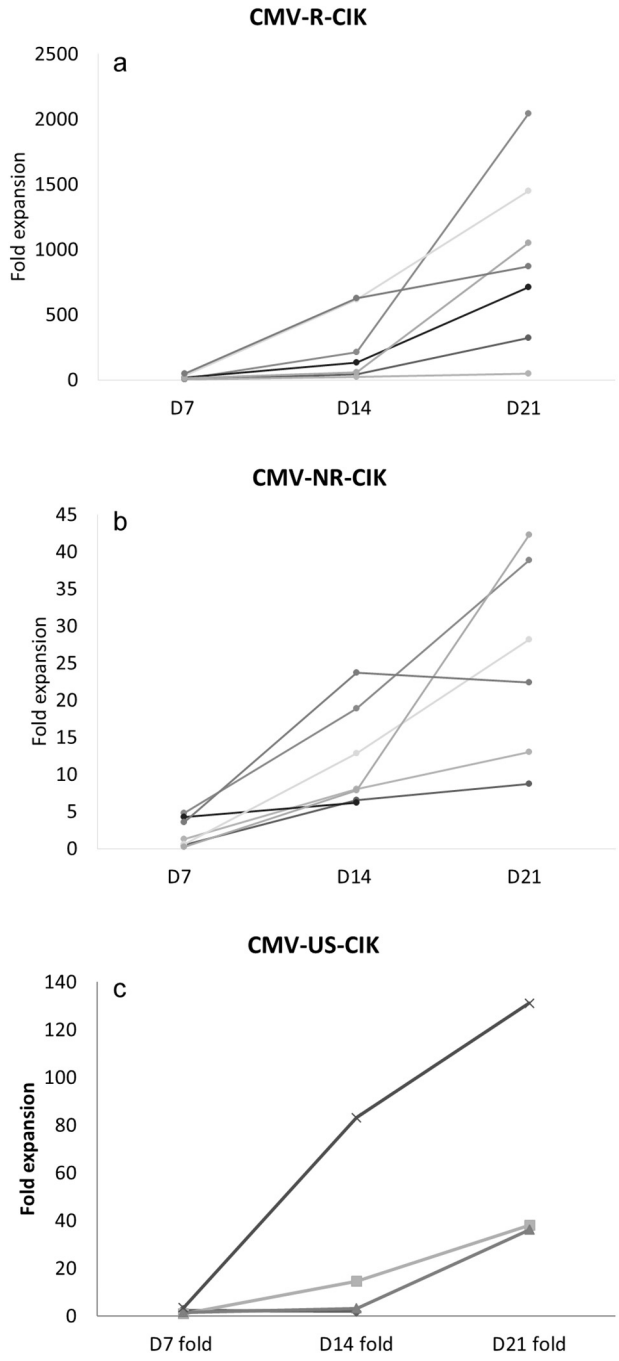
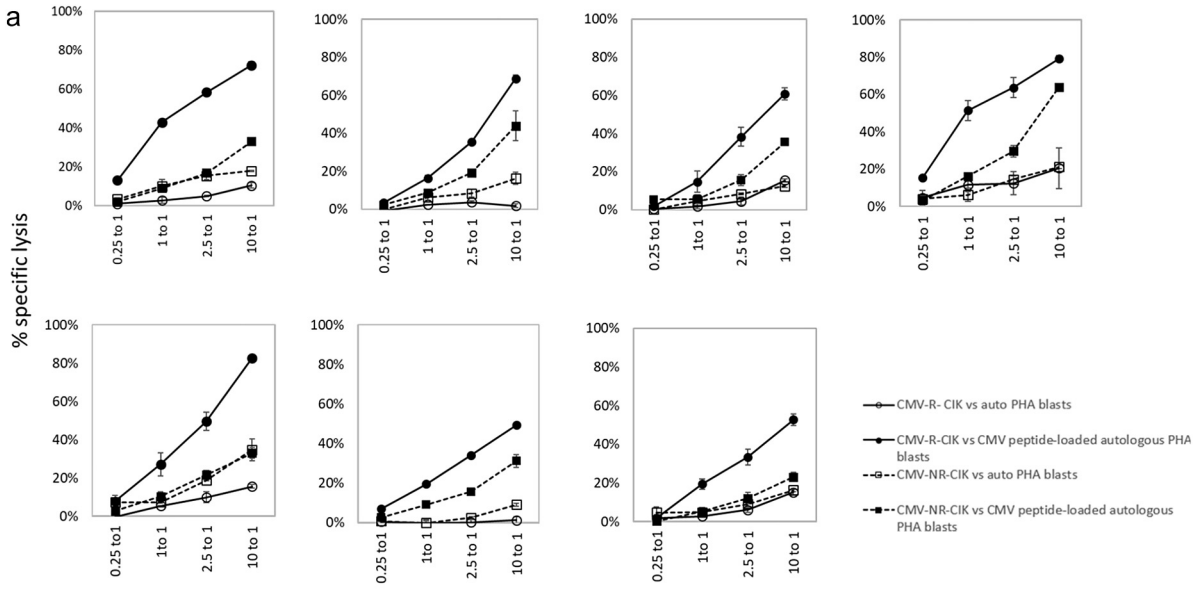
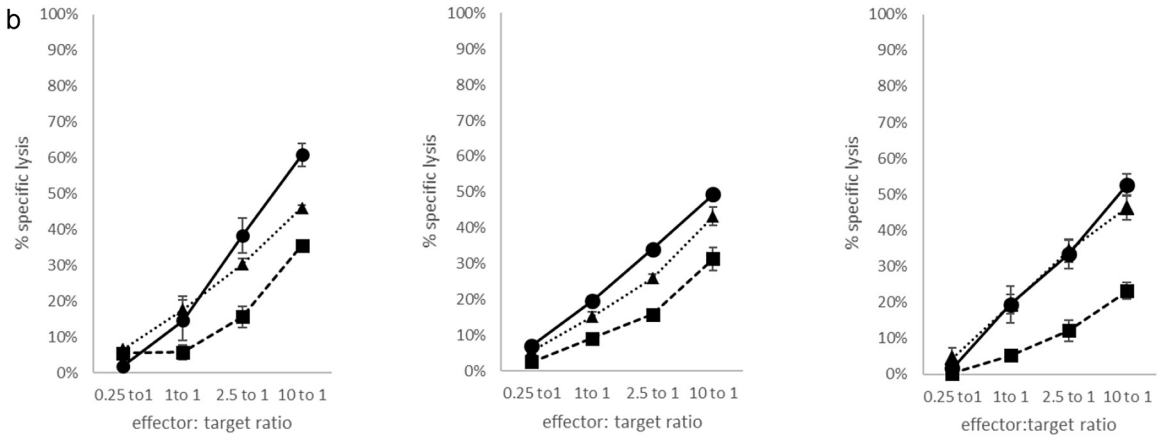


Fig. 1. Fold expansion of CIK compared to cell number seeded in flask, of (a) CMV-R-CIK; (b)CMV-NR-CIK and (c) CMV-US-CIK, showing highest expansion of CMV-R-CIK followed by CMV-US-CIK, and lowest expansion of CMV-NR-CIK.

large inter-sample variation, expansion of CIK was robust as expected from previous experience of CIK culture. There was a great difference in the fold expansion between CMV-R-CIK and CMV-NR-CIK from the same sample done in parallel. At D14 of culture, we observed a median of 131-fold expansion (range 22–628 fold) in CMV-R-CIK cultures, which increased to a median of 872 fold (range 46–2043 fold) by D21 (Fig. 1a) over the starting cell number at culture. In contrast, there was only a median of 8-fold expansion (range 6–24 fold) of CMV-NR-CIK at D14 and 25 fold (range 9–43 fold) after 21 days of culture (Fig. 1b). The difference is statistically significant by Wilcoxon signed rank test ($p < 0.05$ at both D14 and D21).

In 3 out of these 7 samples, a third culture was done in parallel with peptide stimulation and CD137 co-simulation but left unsorted,





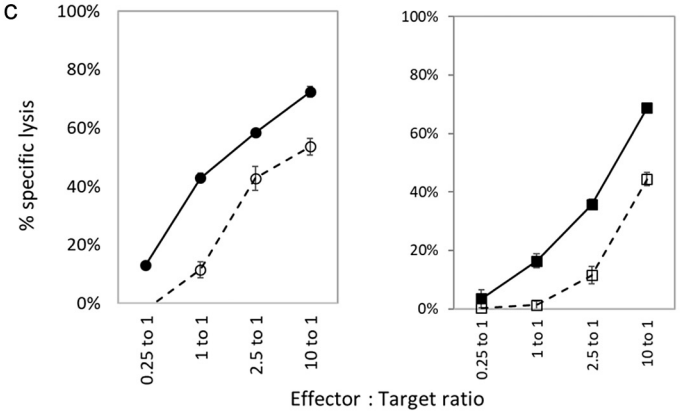


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Fig. 2. a. Cytotoxicity by chromium release assay comparing CMV-R-CIK and CMV-NR-CIK against CMV pp65 and IE-1 pooled peptides loaded autologous PHA blasts. Control target cells are autologous PHA blasts without CMV peptides. Data is expressed as a mean percentage ± SD of triplicates. CMV-R-CIK consistently kills better than CMV-NR-CIK against peptide-loaded autologous PHA basts, while killing against non-loaded PHA blasts is consistently below 20%. The 7 graphs show killing assay of CMV-R-CIK and CMV-NR-CIK cultures from 7 separate samples. b. Cytotoxicity by chromium release assay of CMV-R-CIK, CMV-NR-CIK and CMV-US-CIK against CMV peptide-loaded autologous PHA targets done in parallel. CMV-R-CIK has the highest CMV-specific killing, followed by CMV-US-CIK, while CIM-neg-CIK has the lowest killing. Results of cultures done on 3 separate samples. c. Comparison of cytotoxicity between fresh and frozen-thawed CMV-R-CIK, showing about 20% lower cytotoxicity of frozen-thawed CIK as compared to fresh CIK.

designated as CMV-US-CIK. These cultures showed a median of 15-fold expansion (range 3–82 fold) after 14 days of culture and a median of 38 fold (range 36–130 fold) expansion over starting cell number after 21 days of culture, thus falling between that of CMV-R-CIK and CMV-NR-CIK (Fig. 1c).

3.2. In vitro cytotoxicity against CMV targets

CMV-R-CIK, CMV-NR-CIK and CMV-US-CIK were harvested at between D14 -D21 of culture and cytotoxicity was evaluated by chromium release assay. CMV specificity of the CMV-R-CIK was consistently demonstrated by the killing of CMV pp65 and IE-1 peptides loaded autologous PHA blasts, but minimally against autologous PHA blasts without CMV peptides. Such CIK were highly potent with lysis of at least 33% at E: T ratio of 2.5:1, reaching as high as 83% at ratio of 10:1. The median cytotoxicity at E: T of 2.5:1 was 38% (range 33% to 64%) and at 10:1 was 69% (range 49%–83%). In comparison, CMV-NR-CIK killed more poorly, with the corresponding median cytotoxicity at E: T of 2.5:1 of 17% (range 12% to 30%) and at 10:1 of 33% (range 23%–64%) (Fig. 2a). Comparison of % cytotoxicity between the 7 pairs of CMV-R-CIK and CMV-NR-CIK gave p values of < 0.05 for all 3 E: T ratios, supporting the visual impression of the significant superiority of CMV-R-CIK in CMV-specific killing over CMV-NR-CIK.

Amongst the 3 samples where CMV-US-CIK cultures were done in parallel with CMV-R-CIK and CMV-NR-CIK, it was consistently shown that CMV-R-CIK displayed the highest killing, followed by CMV-US-CIK, while CMV-NR-CIK displayed the lowest killing (Fig. 2b).

We also studied the potency of cytotoxicity after freezing/thawing processes. An aliquot of CMV-R-CIK was cryopreserved, thawed 2 days later, recovered overnight in IL-2, and compared with its fresh counterpart that was continued in culture. Two sets of cryopreserved-thawed CMV-R-CIK showed anti-CMV specificity with slightly reduced potency compared to its fresh counterpart. Fresh CMV-R-CIK showed a 72% and 69% lysis of CMV peptides loaded targets at E: T ratio of 10:1, while that after cryopreservation and thawing was 54% and 44% respectively (Fig. 2c).

3.3. Quantitation and comparison of CMV-reactive T cells within CMV-R-CIK and CMV-US-CIK

The anti-CMV activity of CIK can be measured by the up-regulation of the degranulation marker CD107a upon re-stimulation with pooled peptides. This showed a high proportion of CMV-specific cells in the cultures, with 20.3% - 52.04% of CMV-R-CIK up-regulating CD107a in response to CMV peptide challenge (Fig. 3a). In contrast, only a median of 2.69% (range 0% to 6.92%) of CMV-NR-CIK degranulated in response to peptide re-stimulation, corroborating with the findings of chromium release assay. In the 3 sets of cultures where CMV-US-CIK were cultured in parallel with CMV-R-CIK, the % degranulation was 24.49% vs 41.22%, 7.71% vs 23.32%, and 8.97% vs 20.30% respectively, compared to CMV-R-CIK, which is consistent with the observation of a lower level of cytotoxicity of CMV-US-CIK than CMV-R-CIK in chromium release assay.

Further analysis to correlate % CD107a expression with % chromium release of CMV-R-CIK showed a high degree of correlation between the % degranulating cells with % of chromium release at E: T of 10:1 ($r = 0.91$, $p = 0.004$, Fig. 3b), thereby lending credibility to CD107a as a surrogate marker for reactivity against CMV targets.

The flow cytometric measurement of % of cells with CD107a expression provides a direct quantitation of CMV-reactive T cells in the bulk culture, an informative indicator of the proportion of reactive effectors not provided by the chromium release assay which measures the proportion of targets being killed. We rationalize that the total number of CMV-specific cells that can be generated from a given number of starting cells is an important consideration, besides the purity as reflected by % of specific cells in the product. This can be analysed by comparing the number of CD107a + cells in the end product in relation to the starting cell number at thawing, ie fold increment, for each culture done in parallel. As shown in Table 2, as compared to CMV-R-CIK cultures, the CMV-US-CIK cultures consistently yielded a higher fold increment of degranulating cells over starting cell number, for all 3 parallel comparisons. This is in fact not surprising, as the process of sorting while enriching for CD107a positive T cells will invariably cause some cell loss, whereas omitting the process of sorting ensures inclusion of all CD137 positive cells for expansion. Therefore, on a per-culture basis, culture without sorting gave a better yield of absolute number of CMV-CIK compared to culture sorted for CD137 positive fraction.

3.4. Phenotypic characterization of CMV-R-CIK

We studied the memory T cell subsets of CMV-R-CIK at maturity and compared with that of PBMNC at start of culture. The main bulk of CMV-R-CIK consisted of CD3 + CD56- T cell with a fraction (2.67% to 8.66%) co-expressing CD56, and a majority of CD8 (median 84.3%, range 40.56–96.11%) T cells. Both the CD3 + CD56+ and CD8+ subsets in CIK were higher compared to the T cells in PBMNC at start of culture (Fig. 4a and b). The CMV-R-CIK cultures consisted of mainly effector memory T (T_{EM}) cells as defined by the CD45RO + CD62L- phenotype (median 84%, range 20.83–97.14%), and these were late effectors with loss of CD28 (median 75.25%, range 37.33% to 94.13%). This is in contrast to pre-culture T cells which had a significantly lower T_{EM} subset (median 29.44%, range 0.21–52.14%) and a higher proportion of early CD28+ effectors (median 40.97%, range 6.1% to 77.09%) (Fig. 4c).

The T cell receptor repertoire of CMV-R-CIK was studied by flow cytometry analysis of the TCR Vβ families. We observed that CMV-R-CIK is oligoclonal with preferential expansion of a few families (Fig. 5a) and a paucity of other families, compared to the more normal distribution of pre-culture T cells (Fig. 5b). Different CMV-R-CIK cultures showed preferential expansion of different TCR subfamilies.

3.5. Comparison of potency between CMV-CIK and CMV-CTL

We further compared our protocol of generating CMV-US-CIK with published method where CMV-CTL were generated by peptide loading onto MNC followed by expansion with IL4 and IL7, in 3 parallel cultures. The CMV-CTL expanded by IL4 and IL7 comprised of a majority of CD4 T cells (CD4:CD8 ratio of 4.5,7.4 and 44.0), in contrast to CMV-US-CIK which comprised of a higher proportion of CD8 T cells (CD4:CD8 ratio of 1.7, 0.3 and 0.3 respectively). In addition, CD3 + CD56+ subset was higher in CMV-CIK (3.1%, 18.2% and 6.2%) while this was almost non-existent in CMV-CTL (2.0%, 0.1% and 0.1% respectively). Cytotoxicity was significantly higher in CMV-US-CIK compared to CMV-CTL, where CMV-specific killing was 50.7%, 70.8%

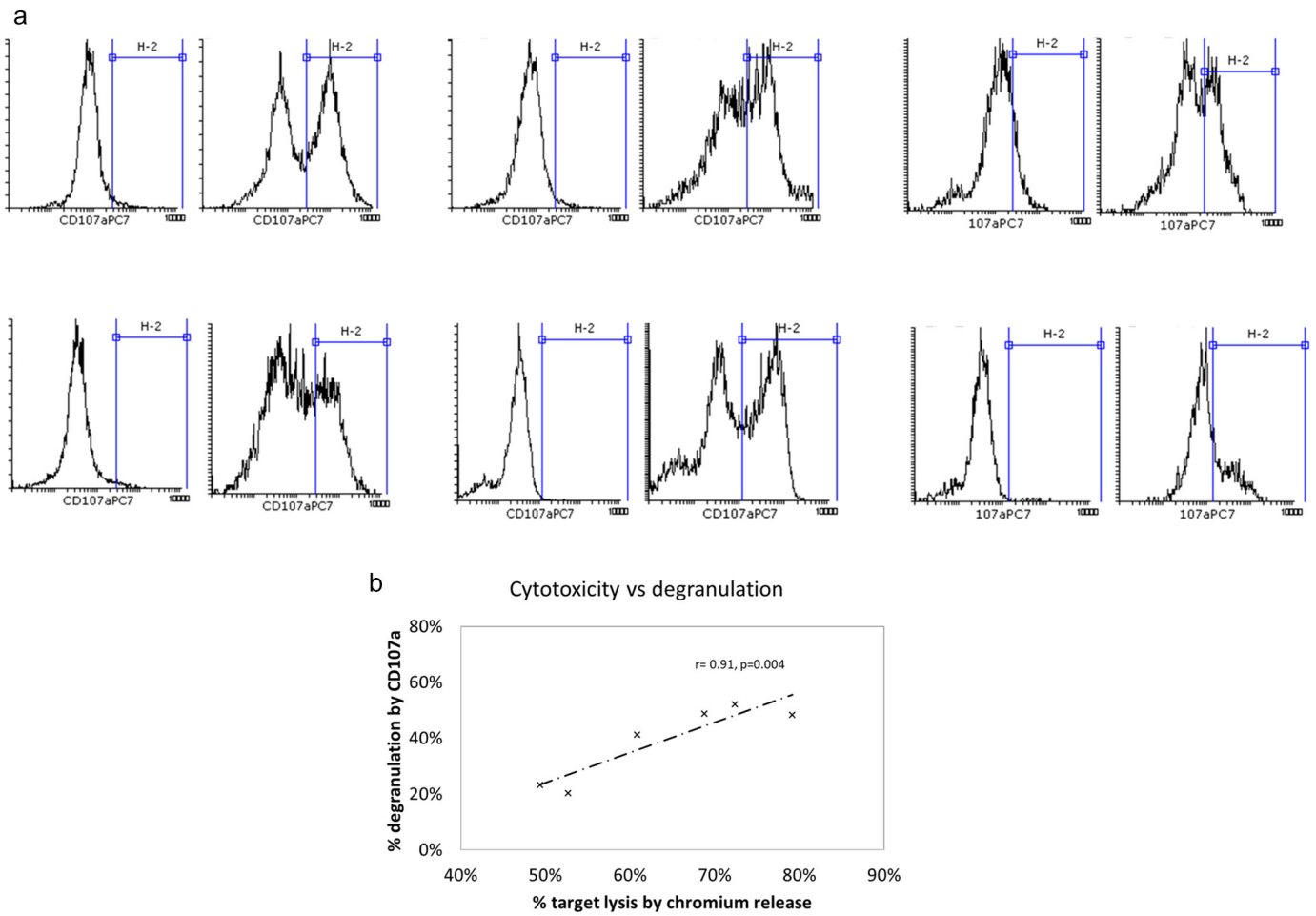

Fig. 3. a. CD107a expression (%) of CMV-R-CIK of 6 pairs of CIK cultures, as measured by flow cytometry upon re-stimulation with CMV peptide. Histogram on the left: control; histogram on the right: after peptide re-stimulation. Nett CD107a expression are 52.04%, 48.53%, 20.3%, 41.62%, 48.94%, 23.32% respectively, from left to right, upper to lower panel. b. Correlation between %CD107a expression by flow cytometry and % specific lysis from chromium release assay done in parallel, for 6 CMV-R-CIK cultures at maturity, showing high coefficient of correlation.

and 49.8% at E;T ratio of 10:1, compared to 32.5%, 16.2% and 4.8% for CMV-CTL (Fig. 6).

4. Discussion

Many studies have demonstrated the safety and efficacy of adoptive T cell therapy in the treatment of virus infection following allo-HSCT. In the earlier days, CMV-specific T cells were clonally expanded with CMV infected fibroblasts [22,23], viral lysate containing CMV proteins [24], or genetically modified APC [6]. These approaches used techniques that involve extensive and prolonged ex vivo manipulation which are not only expensive and time consuming, but also difficult to perform under Good Manufacturing Practice (GMP) conditions. The last few years have seen simplification of methods in generating CMV-CTL. One approach is by direct column-selection of virus-specific T cells that secrete IFN-γ in response to viral peptides [2,4,25] or T cells that stain positive with HLA-peptide tetramers [3], where both methods allow for easy and rapid isolation of CMV-specific T cells. Another recent advancement expands polyclonal CMV-specific T cells by simply stimulating with pooled CMV peptides that span the whole length of the virus antigen followed by expansion with cytokines [8,21]. This latter method using IL4 and IL7 to expand CMV-CTL has been successfully extended to T cells specific for other viruses and multiple viruses in a single culture, and is already in clinical trial showing excellent results in the control of virus reactivation in patients post allo-HSCT [21].

We have been studying the anti-leukemic activity of CIK and have demonstrated feasibility and safety [14] as well as modest clinical efficacy in post- allo-HSCT setting [13]. One deficiency of such CIK is the lack of antigen specificity, being simply a polyclonal expansion of bulk T cells. In this study, we incorporated methods described by others to active, then co-stimulate, followed by the standard CIK expansion protocol to confer CMV specificity to CIK. Firstly, we adopt the method of activation with pooled peptide [8,21] to activate CMV-reactive T cells. Secondly, based on findings well described by others [20,26,27], the upregulated 4–1BB on these T cells upon antigen stimulation is used as a marker to both isolate and co-stimulate these T cells. The ligation of 4–1BB mediates cell proliferation and production of inflammatory cytokines, increases signalling through the T cell receptors and amplifies the cytotoxicity of T cells [20,26]. By the moAb anti-CD137 ligation of 4–1BB on CMV-reactive T cells, it enables not only positive selection but also provides co-stimulation to preferentially expand this tiny fraction of T cells which constitutes only between 0.72% to 5.46% of the total CD3 cells in PBMNC. As is known for positive selection by magnetic column, purity of cell yield is dependent on the frequency of the input cells [28]. Therefore, samples with very low frequency of CD137 positive cells were enriched to a suboptimal purity despite positive column selection (Table 1). Nevertheless, cells thus co-stimulated and selected through their 4–1BB receptor and expanded under CIK condition undergo explosive proliferation as shown in Fig. 1a, in contrast to a much lower expansion of the CMV-NR-CIK counterpart negative for 4–1BB expression cultured under same condition in parallel. As in any biological system, there is marked inter-sample variation in

Table 2
Comparison of generation of CMV-specific cells between 2 methods, with (CMV-R-CIK) and without (CMV-US-CIK) sorting for CD137-reactive T cells before expansion

	CMV-R-CIK					CMV-US-CIK				
ID	Cell count (million)		Degranulation assay			Cell count (million)		Degranulation assay		
	Post thaw (a)	At degranulation (b)	% of degranulating cells (c)	Number of degranulating cells (million) (c * b)	Degranulating cells fold increment over post thaw cells (c * b)/a	Post thaw (a)	At degranulation (b)	% of degranulating cells (c)	Number of degranulating cells (million) (c * b)	Degranulating cells fold increment over post thaw cells (c * b)/a
UPN 5	59.5	136	41%	56.06	0.94	59.50	630	24%	154.29	2.59
UPN 6	72.38	347	23%	80.97	1.12	12.06	202	8%	15.61	1.29
UPN 7	96	305	20%	62.00	0.65	12.00	1416	9%	127.01	10.58

Fig. 4. Comparison between pre-culture PBMNC and post- culture CMV-R-CIK for (a) proportion of CD3 + CD56+ subset, (b) proportion of CD4+ and CD8+ subsets and (c) proportion of memory T cell subsets. Compared to pre-culture PBMNC, CMV-R-CIK consists of a larger CD3 + CD56+ and CD8+ subset, which are late effector memory T cells.

the degree of expansion and function. Despite this, CMV-R-CIK cultures consistently showed a significantly greater cytotoxicity, which is expected since it was expanded from a sorted fraction enriched for T cells reactive to CMV peptides. This consistent observation across 7 different samples gives unequivocal proof of the CMV specificity of CIK expanded with this method. The additional step of co-stimulation likely resulted in the superiority of CMV-CIK over that expanded with IL4 and IL7.

Our protocol was designed with the foremost objective of GMP compliance in methodology to enable clinical translation of the

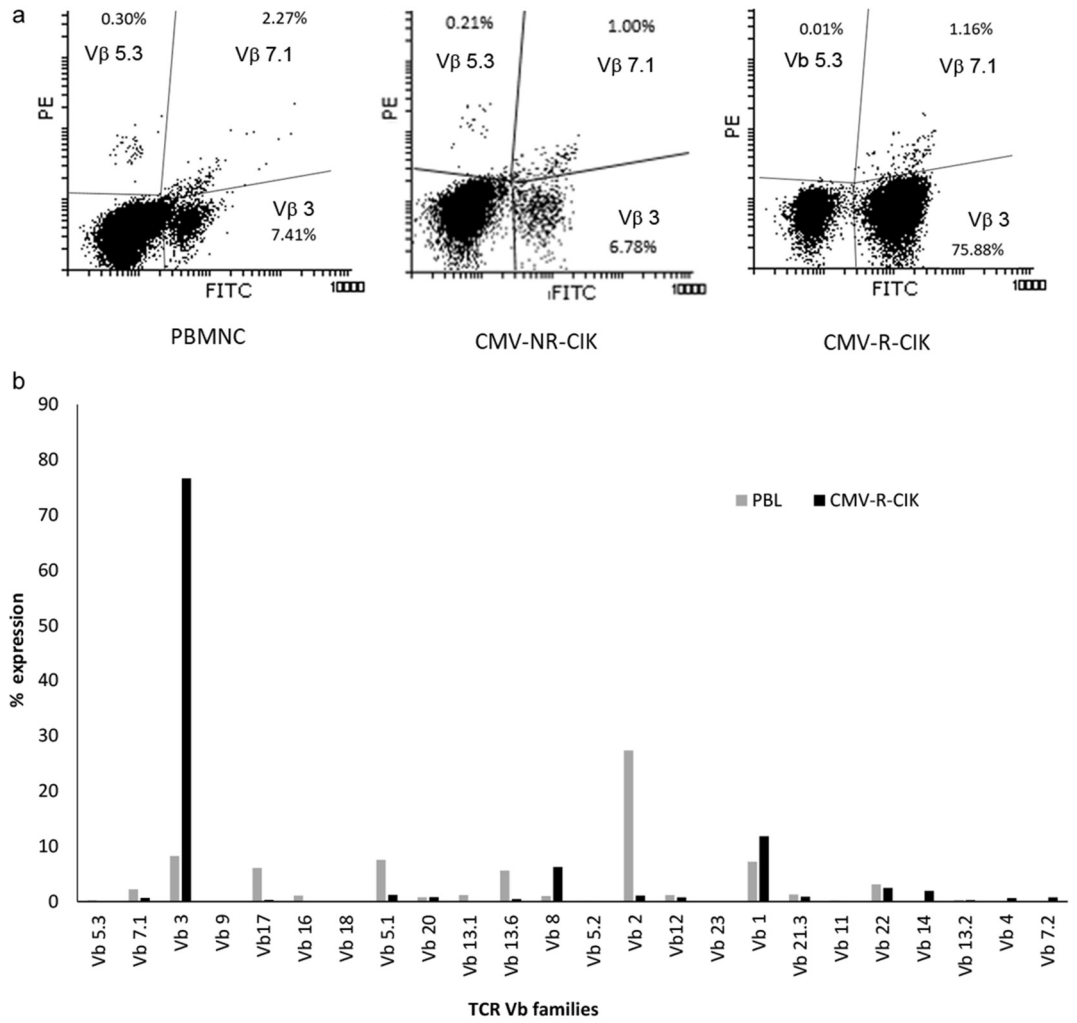

Fig. 5. a. TCR Vβ family analysis of PBMNC and the corresponding CMV-NR-CIK and CMV-R-CIK. This shows an oligoclonal expansion of the Vβ3 family from 7.41% in starting T cells in PBMNC to 75.88% in the CMV-R-CIK, which was not seen in CMV-NR-CIK. b. Distribution of TCR-Vβ family as measured by flow cytometry, of one sample of CMV-R-CIK (gray bars) vs T cells in pre-culture PBMNC (black bars), showing preferential expansion of a few TCR Vβ families in the CMV-R-CIK and a paucity of all the other families, compared to a less skewed repertoire in the pre-culture T cells.

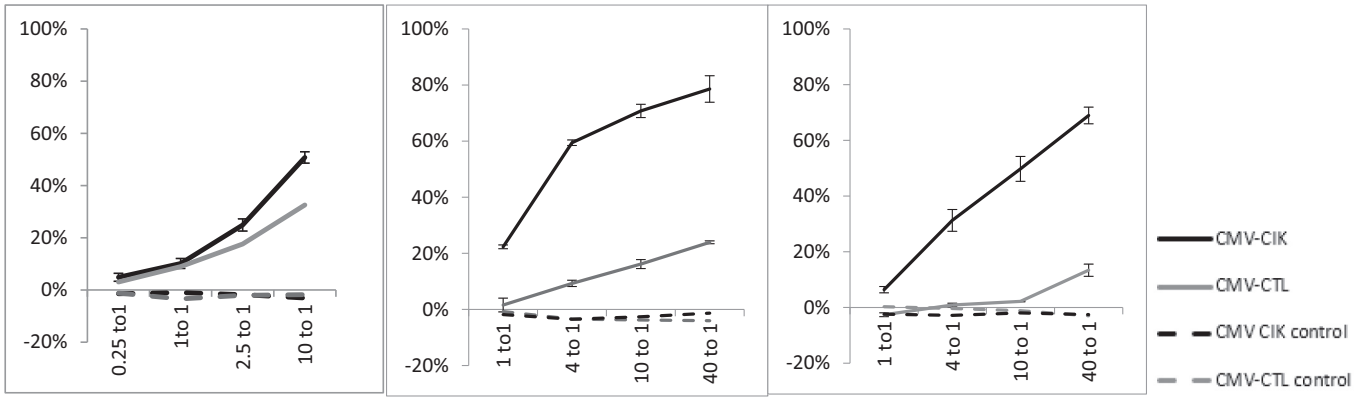

Fig. 6. Cytotoxicity by chromium release assay of CMV-US-CIK compared to CMV-CTL cultured by published method, against CMV peptide-loaded autologous PHA targets done in parallel. CMV-US-CIK has consistently CMV-specific killing compared to CMV-CTL. Results of cultures done on 3 separate samples.

product. Firstly, all essential materials for the production of CMV-CIK including anti-CD137 and the cytokines are available either in GMP-grade or are drugs in clinical use and therefore are GMP-compliant. Secondly, we showed that the method is applicable to frozen thawed PBMNC, allowing flexibility in timing of culture initiation. Very importantly, after proof of principle with positive selection for CD137-expressing PBMNC, we demonstrated that although omission of the column selection step resulted in a product with a lower purity of anti-CMV cells (8–24% as compared to 20–41% degranulate on antigen re-challenge, see Table 2), it in fact generated a higher absolute number of CMV-CIK than that selected for CD137 positivity, on a per-culture basis. This serves to reduce several steps in the manipulation, reduce cost and

bypass issues with GMP-compliance of selection process. We do not expect the lower purity of the CMV-US-CIK (therefore a higher proportion of non-CMV specific T cells in the culture product) to pose any problem in terms of higher alloreactivity. CIK as cultured cells composing largely of memory instead of naïve T cells have been shown in clinical studies to not possess any greater GVHD risk than unmanipulated donor lymphocytes [13,15,16]. Fourthly, we showed a high correlation between % cytotoxicity as measured by chromium release assay and the % degranulating cells as measured by flow cytometry. This provides the basis for using CD107a expression as a non-radioactive and convenient surrogate marker of CMV-specificity which can easily be carried out in the manufacturing facility. Fifthly, we showed that CMV-CIK at maturity can be frozen and thawed without losing too much of the cytotoxicity. This will allow freezing while awaiting sterility clearance, and facilitates flexibility in the timing of infusion, as well as the possibility of its use as a banked product for third party use. Finally, a change in the culture medium from FCS-containing medium to xeno-free, clinical grade medium could similarly yield CMV-US-CIK of equal potency (data not shown).

Our analysis also provided some insight into the characteristics of CMV-CIK generated with this modified method. It is interesting to see oligoclonal expansion of a few TCR Vβ families in the CMV-R-CIK cultures. This is not unexpected, as different CMV-reactive clones utilize different TCR Vβ families for samples with different HLA due to MHC restriction in epitope presentation. We have previously demonstrated that the CIK culture condition results in expansion of polyclonal T cells of a normal distribution of TCR repertoire [12]. The demonstration of oligoclonal expansion corroborates with the observation of CMV-specificity rendered by the simple additional steps of stimulating the antigen-reactive clones with peptides and co-stimulating them through the 4–1BB receptor.

Our protocol results in a preferential expansion of CD8+ T cells at maturity, with a reciprocal decrease in CD4+ T cells. CD8+ T cells are important in controlling viral infections, and immunocompromised patients lacking CD8+ cytotoxic T cells specific for CMV correlates with CMV infection and disease [29,30]. CMV-CIK consists of largely CD45RO + CD62L- effector memory T cells with a predominantly CD27-CD28- late T_{EM} phenotype, which is consistent with antigen-specific T cells generated by other methods used in clinical trials [31]. While terminally differentiated T cells with potent cytotoxicity but without capacity for proliferation and long term persistence may not be the ideal T cell type for tumour immunotherapy [32], this may not be a disadvantage for the treatment of virus infections. Adoptive T cell therapy for virus infection in the post - allo HSCT setting is often used as a bridging measure before immune reconstitution. A few successive infusions till recovery of anti-viral immunity will likely suffice.

Similar efforts in expanding CIK for its CMV specificity has been reported by another group [33] but with some difference. Our method has the initial screening step of identifying donors with CMV-reactive T cells, thus ensuring successful generation of CMV-CIK, in contrast to the 72.2% success rate described. The additional step of co-stimulating the CD137 receptor provides additional signal to potentiate the preferential proliferation and enhance cytotoxicity of the CMV-specific subset. Although measure of CIK function may be difficult to compare due to difference in assay methods, our system results in a similar potency at a lower E: T ratio in cytotoxicity assay (median 38% at E: T of 2.5 to 1, as compared to median of 39.9% at E:T of 5:1 in that reported [33]). We demonstrated in addition that CMV-CIK can be generated from all HLA types besides only HLA-A2 as was studied. This may therefore be an improved method in generating CIK with high CMV specificity.

Compared to CMV-CTL generated by the same pooled peptide but expanded under IL4 and IL7 condition [8,21], we demonstrated in parallel experiments here that CMV-CIK generated with our method is superior in cytotoxicity. Although the cytokine milieu for both methods are known to promote Th1 polarization [8,34], the composition of T cell subsets is different between these 2 culture methods, and the superior cytotoxicity may be explained by the higher proportion of the potent CD3 + CD56+ NK-like T cells, as well as CD8 in the CMV-CIK cultures, which is known to possess more potent cytotoxicity than CD4 subset [12]. There are likely to be other differences in the T cell subsets and expression of receptors that may account for the difference in potency, which are not analysed in this comparison.

5. Conclusion

We have developed a simple and GMP-compliant method to generate a large number of highly specific and potent CMV-CIK within 2–3 weeks of culture by a single exposure to CMV pooled peptides pp65 and IE-1. CIK generated by this method is a promising candidate for clinical use in the treatment of CMV infection after allo-HSCT. Its efficacy has to be verified by clinical studies, either in pre-emptive setting for patients with low level CMV viremia, or in salvage setting for those who fail to respond to anti-viral drugs. This method can potentially be adapted to generate virus specific CIK against a whole range of other viruses and possibly multiple viruses, as long as there is a small fraction of T cells reactive to the virus peptide epitopes which can be co-stimulated and expanded. Further work along this approach is underway.

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Declaration of Competing Interest

The authors have no competing interest to declare.

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