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The immunogenicity of virus-derived 2A sequences in immunocompetent individuals

C Arber[1], **H Abhyankar**[1], **HE Heslop**[1,2,3], **MK Brenner**[1,2,3], **H Liu**[1,4], **G Dotti**[1,2,5], and **B Savoldo**[1,3]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, The Methodist Hospital and Texas Children's Hospital, Houston, TX, USA

[2]Department of Medicine, The Methodist Hospital and Texas Children's Hospital, Houston, TX, USA

[3]Department of Pediatrics, The Methodist Hospital and Texas Children's Hospital, Houston, TX, USA

[4]Department of Biostatistics Shared Resource Dan L. Duncan Cancer Center, The Methodist Hospital and Texas Children's Hospital, Houston, TX, USA

[5]Department of Immunology, Baylor College of Medicine, The Methodist Hospital and Texas Children's Hospital, Houston, TX, USA.

Abstract

Genetic engineering of T cells for adoptive immunotherapy in cancer patients has shown significant promise. To ensure optimal antitumor activity and safety, the simultaneous expression of multiple genes is frequently required, and short viral-derived 2A sequences are increasingly preferred for this purpose. Concerns exist, however, that these virus-derived sequences may induce unwanted immune responses, and thus diminish persistence of the gene-modified cells after adoptive transfer. Whereas such responses were absent in immunocompromised recipients, potential immunogenicity in immunocompetent individuals remains a concern. We now address whether *ex vivo* T cell responses can be elicited against the most widely used 2A sequences (2A-Thosea asigna virus (TAV) or 2A-equine rhinitis virus (ERAV), specifically) in immunocompetent individuals. We used a potent *ex vivo* culture system previously validated to induce T cell responses even against weakly immunogenic antigens. Of the sixteen donors tested, only five released very low levels of interferon-γ in response to 2A-TAV peptide mixtures (single peptide specificity in three donors, adjacent self-antigen peptide specificity in one donor and nonspecific reactivity in one donor). None of them produced cytotoxic activity or responded to 2A-ERAV. These results suggest that exposure to viral-derived 2A sequences is unlikely to produce unwanted T cell responses in immunocompetent individuals and further supports their continued use for studies of human gene therapy.

Keywords

2A sequences; polycistronic vectors; T cell gene transfer; immunogenicity

Correspondence: Professor B Savoldo, Center for Cell and Gene Therapy, Baylor College of Medicine, The Methodist Hospital and Texas Children's Hospital, 6621 Fannin St, MC 3-3320, Houston, TX 77030, USA. bsavoldo@bcm.edu.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

INTRODUCTION

Adoptive T cell-based immunotherapies and vaccines are promising approaches for cancer patients, and genetic engineering can significantly improve their potency.[1,2] Efficient expression of multiple genes in one single polycistronic vector can simultaneously coordinate the expression of multiple components of the immune system against cancer cells and counterbalance tumor immune evasion mechanisms,[3] and is often critical for the success of these therapies. For example, production of T lymphocytes that will safely and efficiently kill tumor cells after adoptive transfer may require simultaneous genetic modifications to increase their tumor specificity by forced expression of engineered T cell receptors;[2,4] enhance their trafficking by expression of chemokine receptors;[4,5] prolong their persistence by forced expression of cytokines or co-stimulatory molecules[6–11] and augment safety by the incorporation of a suicide switch to remove gene-modified cells on demand.[12,13]

Of the available strategies to generate polycistronic vectors, virus-derived 2A sequences are one of the most effective.[14] These sequences are *cis*-acting hydrolase elements that mediate a ribosomal skip between 2A-linked genes,[15] resulting in stoichiometric protein production[16] that can improve transgene expression and function, for example, of T cells engineered with transgenic αβT cell receptors.[17] Moreover, 2A sequences are quite short (54–60 nucleotides), and therefore have little impact on vector-packaging limits. However, as the 2A sequences are of viral origin, their expression in fusion proteins could lead to the expression of immunogenic epitopes and thereby diminish the *in vivo* persistence of corresponding gene-modified cells.

Recently, 2A sequences have been successfully incorporated in vectors used in human studies without eliciting discernible immune responses, although the recipients in these trials were significantly immunocompromised.[13,17] Thus, to discover whether virus-derived 2A sequences may cause troublesome immunogenicity in immunocompetent individuals, we assessed if T cell responses could be elicited to protein regions derived from two vectors containing either the 2A-TAV (Those asigna virus-derived)[13] or the 2A-ERAV (equine rhinitis virus-derived)[7] sequences. We used a potent *ex vivo* culture system that has been previously optimized to expand T cells with specificity for weak antigens, even from antigenically naive individuals,[18] such as umbilical cord blood T cells.[19] Our results support the continued exploration of 2A sequences even in immunocompetent human subjects.

RESULTS AND DISCUSSION

Using our optimized culture system,[18–20] we successfully generated T cell lines from normal adult donors that had robust interferon-γ (IFN-γ) production in response to the cytomegalovirus-derived pp65 peptide mixture (pepmix) (1274.6±91.9 IFN-γ spot-forming cells (SFCs) per 10^5 cells) in 7/7 donors (Figure 1a) and to the weak tumor-associated antigen, preferentially expressed antigen of melanoma (PRAME) pepmix[18] (409.1±29.7 IFN-γ SFCs per 10^5 cells) in 8/9 donors (Figure 1b). By contrast, when we used the same culture conditions with peptide mixtures derived from the two 2A sequences, we found minimal reactivity against the 2A-TAV pepmix in only 5 of the 16 donors tested (59.6±6.7 IFN-γ SFCs per 10^5 cells) (Figure 1c), and no discernible responses against the 2A-ERAV-pepmix in any of the 11 donors tested (Figure 1d). Phenotypically, all lines were a mixture of $CD4^+$ and $CD8^+$ cells, and contained some natural killer cells, with a trend (P=0.07) for a lower proportion of $CD8^+$ cells in the 2A-TAV and 2A-ERAV lines (for 2A-TAV: $CD8^+$=48.6±23.1%; for 2A-ERAV: $CD8^+$=48.5±20.32%), as compared with pp65-specific T cell lines ($CD8^+$=70.4±12.8%) or with PRAME-specific T cell lines ($CD8^+$=58.8±19.7%, Figure 2).

To identify the weakly immunogenic peptides derived from the 2A-TAV sequence, we tested each of the 11 single 15-mer peptides against the five weakly reactive T cell lines. Three of these five lines (derived from donor nos. 13, 14 and 15, Figure 3a) released IFN-γ upon exposure to the RAEGRGSLLTCGDVE peptide (51.9±6.9 IFN-γ SFCs per 10^5 cells, peptide no. 5 of the pool, Figure 4a). The line from donor no. 9 (Figure 3a) reacted against the CFNFLRKKLFFKTSA peptide (57.3±10.3 IFN-γ SFCs per 10^5 cells, peptide no. 1 of the pool, Figure 4a) and simultaneously against the PRLLFFLLFLTPMEV peptide (21.0±15.1 IFN-γ SFCs per 10^5 cells, peptide no. 11 of the pool, Figure 4a). These are junctional peptides between the 2A sequence, and the carboxyand amino-terminal sequences of the expressed genes of interest (human inducible caspase9 and the signal peptide sequence of human CD19, respectively). The line derived from donor no. 8 was responsive against the 2A-TAV pepmix, but had no discernible reactivity to any of the individual peptides contained in the mixture and was thus classified as nonspecific.

To discover the associated biological consequences of the low-level IFN-γ responses seen in the four antigen-specific T cell lines, we measured their cytotoxic properties using the CD107a/b degranulation assay. All these lines responded appropriately to polyclonal stimulation with phorbol myristate acetate and ionomycin (66±13% CD107a/b$^+$ CD3$^+$ cells), and therefore had functional potential, but no specific degranulation was detected upon stimulation with the 2A-TAV pepmix (2.5±0.5% CD107a/b$^+$ CD3$^+$ cells) or the specific single peptide no. 5 (1.7±0.5% CD107a/b$^+$ CD3$^+$ cells for lines from donor nos. 13–15), as compared with the negative control (2A-ERAV-pepmix, 2.2±1% CD107a/b$^+$ CD3$^+$ cells) (Figures 3b and c, P=NS), consistent with the minimal reactivity shown in the IFN-γ ELISpot assay (Figures 1c and 3a). As expected, pp65-specific- and PRAME-reactive lines specifically degranulated on exposure to the corresponding pp65 or PRAME pepmixes (13.3±12.2% CD107a/b$^+$ CD3$^+$ cells, n=9), but not to control peptides (2A-ERAV-pepmix, 2.7±1.4% CD107a/b$^+$ CD3$^+$ cells) (Figures 3b and c, P=0.03).

We next assessed whether T cells transduced with the 2A-TAV-containing retroviral vector, SFG.iCasp9.2A-TAV.ΔCD19, could present and process any of these epitopes. Autologous T cells from the reactive donor nos. 9, 13, 14 and 15 were transduced (CD3$^+$CD19$^+$=53.7±11.5%) (Figure 3d) and then used as targets in a standard 6-h ^{51}Chromium (Cr)-release assay. No significant lysis of 2A-TAV transduced (TD) or non-transduced autologous target T cells was observed in any of the donors tested (3.5±2.9% specific lysis against NT, 3.75±3.9% specific lysis against TD T cells, effector to target ratio (E:T) 20:1) (Figure 3e).

Thus, our potent *ex vivo* culture system shows that the viral-derived 2A-TAV and 2A-ERAV ribosomal skip sequences, and adjacent fusion protein regions exhibit very low (n=5) to absent (n=11) immunogenicity in 16 healthy donors. That this paucity of response is genuine rather than a reflection of deficiencies in our detection system is demonstrated by the anticipated high level of reactivity against cytomegalovirus-derived antigens (7/7) or weak tumor antigens (8/9), and by our ability to generate an expected low frequency (1/16) of lines weakly reactive with peptides derived from the self-antigens linked by the 2A sequences (human caspase9 and CD19) that were also included in the peptide mixtures. Of note, the three donors with low-level reactivity to the peptide no. 5 of the 2A-TAV sequence (donors no. 13–15) shared the same class-I-restricted human leukocyte antigen (HLA) alleles (HLA-A*02 and HLA-B*44) and certain class-II haplotypes, including HLA-DRB1*04, HLA-DQB1*03 (Table 1). These findings suggest that the reactive peptide no. 5 of the SFG.iCasp9.2A-TAV.ΔCD19 retroviral vector could contain an HLA class-I or class-II epitope presented in the context of either of these HLA molecules. Given the overall low level of reactivity of these lines, we could not further dissect these reactivities, and precisely identify the epitope and its HLA context. The epitope prediction algorithms from

SYFPEITHY (http://www.syfpeithi.de/) and the BioInformatics and Molecular Analysis Section, NIH, (http://www-bimas.cit.nih.gov/molbio/hla_bind/) failed to indicate a significantly high-scoring epitope within the 2A-TAV sequence in the context of HLA-A*0201, HLA-A*0205, HLA-B*4402, HLA-B*4403 or HLADRB1*0401 (data not shown, HLA-DQB1 epitopes are not available for prediction). Instead, the only epitope scoring significantly high in both prediction algorithms in the context of the HLA-A*0201 molecule was contained within the sequence of peptide no. 11 of the SFG.iCasp9.2A-TAV.ΔCD19 retroviral vector, which was recognized by the line derived from the HLA-A*02^+ donor no. 9, and corresponded to the signal peptide of the human CD19 self-antigen (Figures 3a and 4a). Nonetheless, 7 of the 11 HLA-A*02^+ donors failed to generate responses against such epitopes.

Adoptive T cell therapy in cancer patients aims to generate a long-term persistence of the transferred gene-modified T cells. This can be undermined if vectors contain potentially immunogenic sequences, and TD cells are infused in immuno-competent individuals or if multiple T cell administrations are performed. In these situations, *in vivo* sensitization against vector components can be induced and result in the immune-mediated elimination of the TD cells. No *ex vivo* system can fully reproduce the *in vivo* situation of a 'booster vaccination' effect, but we have attempted to answer immunogenicity concerns in our *in vitro* culture system by using three antigen-specific stimulations with professional antigen-presenting cells (dendritic cells) to amplify T cell responses from immunocompetent individuals. No significant cytotoxic activity was observed in the 16 donors tested, although this limited sample size means we cannot exclude a true positive rate of up to 0.17.

Only clinical trials that include these 2A sequences in immunocompetent individuals can definitely address the issue of their immunogenicity. However, the minimal reactivity we observed to the 2A-TAV sequence (in terms of IFN-γ production) associated with the lack of cytotoxic activity, as measured by the CD107a/b degranulation, seems unlikely to result in significant biological consequences, particularly as we found no evidence that these peptides could even be naturally processed and presented by T cells. In conclusion, our data suggest that the incorporation of 2A sequences in polycistronic vectors should not precipitate unwanted immune responses against the TD cells. Of the two 2A sequences studied, 2A-ERAV may be even less immunogenic than 2A-TAV. Careful monitoring for potential immunogenicity in future clinical trials with diverse patients and vectors will, however, still be required before we can be certain that there is no effective 2A-directed immune response against the TD T cells.

MATERIALS AND METHODS

Peptides and pepmixes

Fifteen-mer peptides overlapping by 11 amino acids spanning the 2A-TAV peptide sequence region of the SFG.iCasp9.2A-TAV.ΔCD19 retroviral vector[21] (Figure 4a) and the 2A-ERAV peptide sequence of the SFG.iCasp9.2A-TAV.CAR-CD19-28ζ.2A-ERAV.IL (interleukin)-15 retroviral vector[7] (Figure 4b) were synthesized by JPT Peptide Technologies (Berlin, Germany). Lyophilized peptides were reconstituted in dimethyl sulfoxide and then pooled into peptide mixtures (pepmixes) containing all the 11 peptides (2A-TAV mix or 2A-ERAV mix; 10 mg ml^{-1}) or stored as single peptides (10 mg ml^{-1}). The pepmixes or single peptides were used to pulse dendritic cells, as previously described.[18] Pepmixes spanning the cytomegalovirus pp65 protein or the cancer testes antigen PRAME (JPT Peptide Technologies) were used as controls.[18,20]

Generation and expansion of peptide-specific T cell lines

Buffy coats from healthy volunteer blood donors were obtained through the Gulf Coast Regional Blood Center, Houston, TX, USA. HLA typing of these samples was performed by the HLA, Flow and Diagnostic Immunology Laboratory of the Department of Laboratory Medicine at The Methodist Hospital, Houston, TX, USA, using PCR-SSO DNA-based procedures. Peripheral blood mononuclear cells were isolated by Lymphoprep (Accurate Chemical and Scientific Corp, Westbury, NY, USA) density gradient centrifugation. Dendritic cells were generated as previously described,[18] starting from CD14-selected cells and, after maturation, pulsed with 5 μ_M of the specific pepmix for 2 h at 37 °C and finally used to stimulate peripheral blood mononuclear cells at an E:T ratio of 20:1 in complete media, containing 45% Click's media (Irvine Scientific, Santa Ana, CA, USA), 45% RPMI 1640 (HyClone Laboratories, Logan, Utah), 5% heat-inactivated human AB serum (Valley Biomedical, Winchester, VA, USA), 1% L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in the presence of a previously validated combination of cytokines (IL-7 (10 ng ml^{-1}), IL-12 (1 ng ml^{-1}) and IL-15 (2 ng ml^{-1}) (all from Peprotec, Rocky Hill, NJ, USA or R&D Systems, Minneapolis, MN, USA).[18] On days 9 and 16 of culture, T cells were re-stimulated with pepmix-pulsed dendritic cells at an E:T ratio of 10:1 in media containing IL-7, IL-12 and IL-15. IL-2 (50 U ml^{-1}) was added to the culture from day 16.

Retroviral transduction

The retroviral supernatant encoding the SFG.iCasp9.2A-TAV.ΔCD19 vector was prepared as previously described.[13] Peripheral blood mononuclear cells from donors 9, 13, 14 and 15 were activated with immobilized OKT3 and anti-CD28 (BD) antibodies, and IL-2 100 U ml^{-1}. After 72 h, cells were transduced on retronectin-coated plates (Takara Bio Inc, Shiga, Japan) as previously described.[7] Phenotype of TD cells was assessed by FACS 3 days after transduction, and used as targets in ^{51}Cr assays.

Immunophenotyping

T cell subset distribution was analyzed after the third stimulation by staining with fluorochrome-conjugated antibodies for CD3–APC, CD4–PE, CD8–PerCP and CD56–FITC. Transduction efficiency was analyzed by staining cells with CD3–FITC and CD19–APC antibodies. All antibodies were from BD Biosciences, San Jose, CA, USA, or Beckman Coulter, Brea, CA, USA; data acquisition was performed on a FACSCalibur (BD Biosciences) using CellQuest Software (BD Biosciences) and data analysis was performed using FlowJo Software (Treestar, Ashland, OR, USA). To evaluate CD107a/b degranulation, T cells at the end of the third antigen-specific stimulation were resuspended at 1×10^6 cells ml^{-1} in complete media with 10% fetal bovine serum (HyClone) without cytokines. After blocking protein transport (GolgiPlug and GolgiStop, both from BD), following the manufacturer's instruction, cells were stained with FITC-conjugated CD107a and CD107b (both from BD), and then stimulated with either relevant or irrelevant pepmixes or single peptides (5 μ_M) for 4–5 h at 37 °C at 5% CO_2. Phorbol myristate acetate (25 ng ml^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and ionomycin (1 μg ml^{-1}, Sigma-Aldrich) were used as positive controls. Cells were then collected, fixed for 10 min in 1% Cytofix (BD), washed and then stained for 30 min with CD3–APC, CD4–PE and CD8–PerCP or appropriate isotype controls. After washing, cells were analyzed on the FACSCalibur.

ELISpot assay

The IFN-γ ELISpot assay was performed as previously described.[18] In brief, 1×10^5 T cells per well were plated in triplicates and then stimulated with 5 μ_M of the specific pepmixes of the single peptides or media alone. As positive control, T cells were stimulated with 25 ng

ml^{-1} of phorbol myristate acetate and 1 mg ml^{-1} of ionomycin. The IFN-γ SFCs were enumerated (ZellNet, Fort Lee, NJ, USA). T cell lines were defined as being reactive when the number of SFCs was ≥2× above background level (media control or irrelevant pepmix).

Chromium-release assay

The cytotoxic activity of T cells was evaluated using a standard 6-h ^{51}Cr-release assay, as previously described.[18] After labeling with ^{51}Cr, target cells were incubated with T cells at different E:T ratios in medium alone or in 1% Triton X-100 (Sigma-Aldrich) to determine spontaneous and maximum ^{51}Cr-release, respectively. The mean percentage of specific lysis of triplicate wells was calculated as follows: ((test counts – spontaneous counts)/(maximum counts – spontaneous counts)) × 100%.

Statistical analysis

Data were summarized by means and s.d. Student's *t*-test was used to determine the statistically significant differences between the samples, with *P*-value <0.05 indicating a significant difference. When multiple comparison analyses were required, statistical significance was evaluated by a repeated measures ANOVA, followed by a Newman–Keuls test for multiple comparisons. The exact binomial method was used to calculate the confidence interval.

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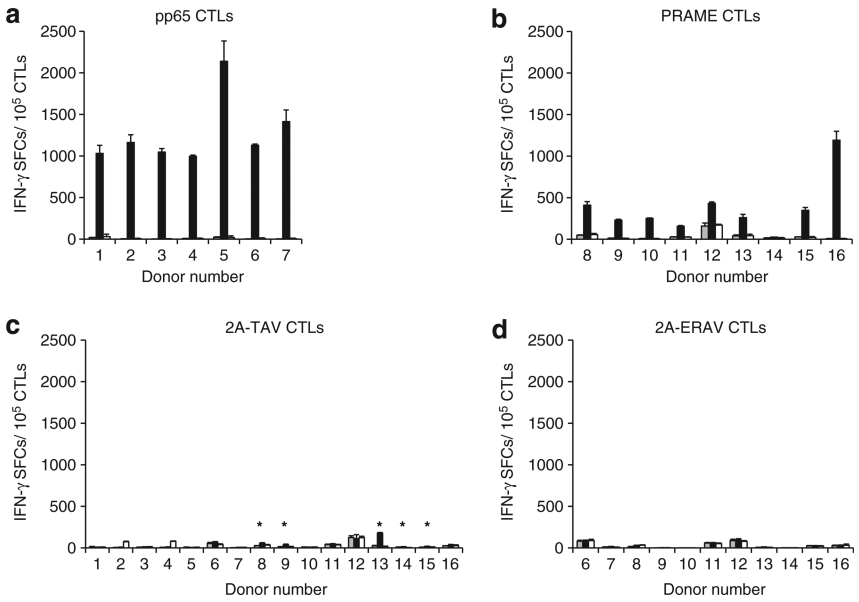

Figure 1.

T cell lines produce IFN-γ upon antigenic stimulation. After three antigen-specific stimulations with pepmix-pulsed dendritic cells, T cell lines were analyzed for IFN-γ production by ELISpot in response to media (gray bars), relevant pepmix (black bars) or irrelevant pepmix (white bars). IFN-γ SFCs per 1×10^5 CTLs from (**a**) cytomegalovirus (CMV) pp65 lines, (**b**) PRAME lines, (**c**) 2A-TAV lines and (**d**) 2A-ERAV lines are shown (mean±s.d. of triplicates). The 2A-TAV lines from donors nos. 8, 9, 13, 14 and 15 were reactive against the 2A-TAV pepmix with SFCs ≥2× above media or irrelevant pepmix (*), and therefore selected for further analyses.

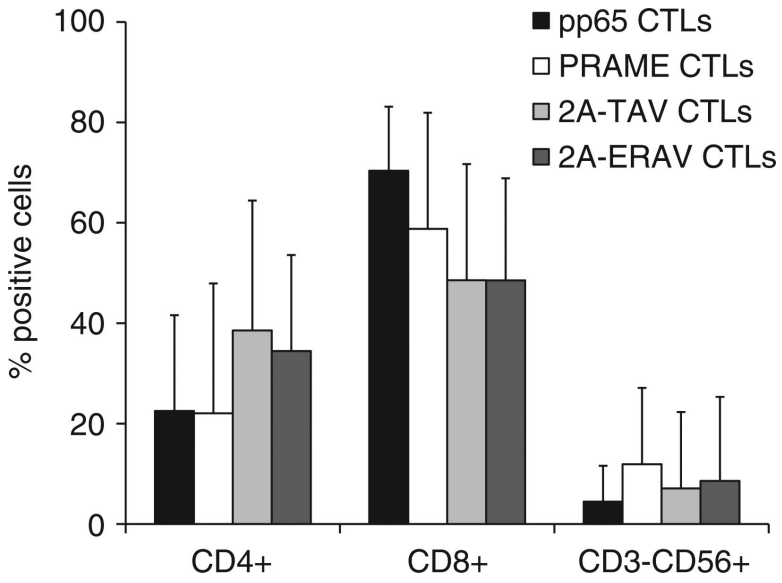

Figure 2.

T cell lines contain a mixture of $CD4^+$ and $CD8^+$ T cells. The phenotypes of the T cell lines were analyzed by flow cytometry after three antigen-specific stimulations. All lines contained a mixture of $CD4^+$ and $CD8^+$ T cells, with some admixed $CD3^-CD56^+$ NK cells. Shown are mean±s.d. of percentage of positive cells for CMV-pp65 lines (n=7, black bars), for PRAME lines (n=7, white bars), for 2A-TAV lines (n=14, light gray bars) and for 2A-ERAV lines (n=9, dark gray bars). We found a trend for a lower proportion of $CD8^+$ T cells in 2A-TAV and 2A-ERAV lines (P=0.07) as compared with CMV-pp65 or PRAME lines.

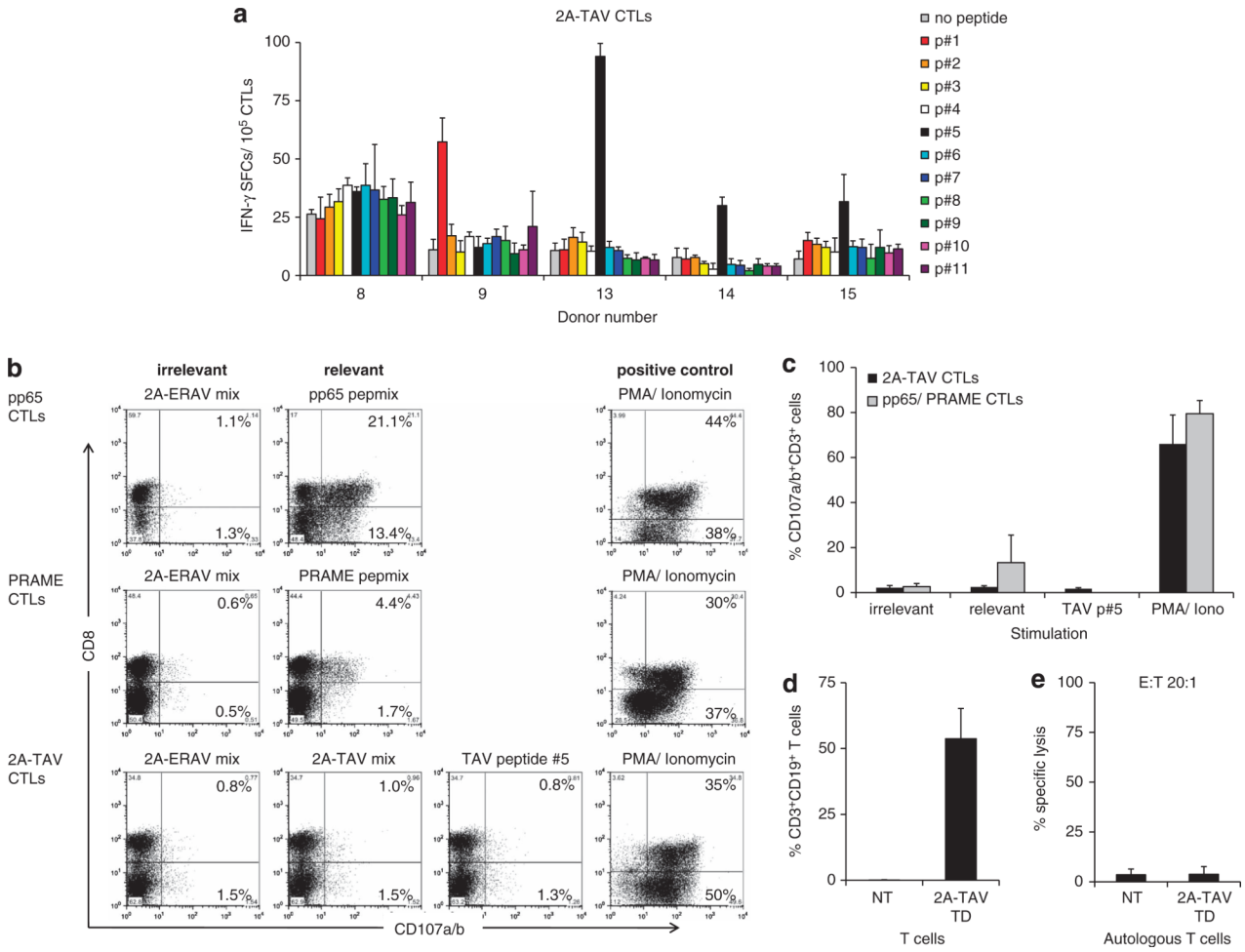

Figure 3.

Lack of cytotoxic activity of 2A2A-TAV-specific T cells despite low levels of IFN-γ production. (**a**) The 2A-TAV pepmix-reactive T cell lines from donors nos. 8, 9, 13, 14 and 15 were analyzed by IFN-γ ELISpot against the single 15-mer peptides contained in the 2A-TAV pool (peptides p no. 1-p no. 11, colored bars) or no peptide (gray bar, mean±s.d. of triplicates). Weak reactivity was found in 2A-TAV lines against the shown single peptides. Donor no. 8 only showed nonspecific reactivity. (**b**) Representative FACS plots of the CD107a/b degranulation assay (gated on $CD3^+$ cells) from a pp65-specific CTL line, a PRAME-specific CTL line and a 2A-TAV-specific CTL line stimulated with irrelevant (2A-ERAV) or relevant (pp65, PRAME or 2A-TAV) pepmixes, 2A-TAV peptide no. 5 (p no. 5) or phorbol myristate acetate (PMA)/ionomycin (positive control). (**c**) Summary of CD107a/b degranulation assay for 2A-TAV CTLs (n=4) and pp65/PRAME CTLs (n=9) (mean±s.d. of % $CD107a/b^+CD3^+$ cells). (**d**) Summary of transduction efficiency of SFG.iCasp9.2A-TAV.ΔCD19-TD T cells from donors no. 9, and 13–15 (mean±s.d. of % $CD19^+CD3^+$ cells). (**e**) Summary of ^{51}Cr-release assay of 2A-TAV-specific CTLs from donors no. 9, and 13–15 tested against non-transduced (NT) or 2A-TAV TD autologous T cells (mean±s.d. of % specific lysis) at an E:T ratio of 20:1.

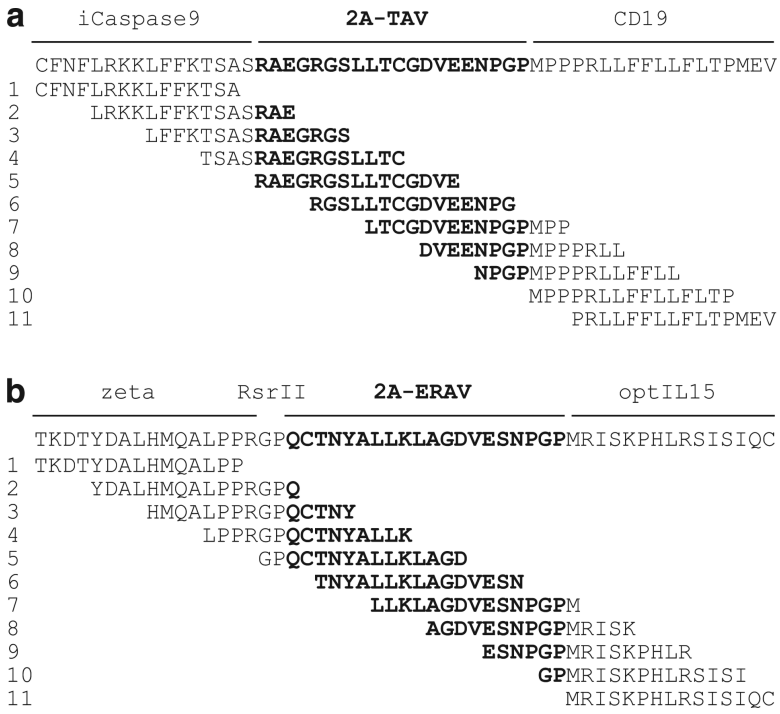

Figure 4.

2A sequences are contained in polycistronic retroviral vectors. (**a**) The 2A-TAV sequence and the single overlapping 15-mer peptides (no. 1–11) were derived from the SFG.iCasp9.2A-TAV.ΔCD19 retroviral vector.[13] (**b**) The 2A-ERAV sequence and the single overlapping 15-mer peptides (no. 1–11) were derived from the SFG.iCasp9.2A-TAV.CAR19-28ζ.2A-ERAV.optIL15 retroviral vector.[7]

Table 1

Donor HLA typing

HLA/donor	HLA-A2 FACS	HLA-A	HLA-B	HLA-DRB1	HLA-DQB1
1	Pos	na	na	na	na
2	Pos	na	na	na	na
3	Neg	na	na	na	na
4	Pos	*02, —	*07,*15	*13, —	*03, *06
5	Pos	*02, *03	*51, —	*04, *07	*02, *03
6	Neg	na	na	na	na
7	Neg	na	na	na	na
8	Pos	*02, *03	*07, *35	*07, *09	*02, —
9	**Pos**	***02, *23**	***07, *18**	***11,*13**	***03, *06**
10	Pos	*02 *68	*07, *51	*04, *15	*03, *06
11	Pos	*02, —	*42, *53	*01, *08	*04, *05
12	Pos	*02, *33	*42, *58	*11, *13	*03, *06
13	**Pos**	***02, *03**	***14, *44**	***04, —**	***03, —**
14	**Pos**	***02, *23**	***44, *51**	***04, *14**	***03, *05**
15	**Pos**	***02, *29**	***44, —**	***03, *11**	***02, *03**
16	Pos	*02, —	*35, —	*04, *08	*03, *04

Abbreviations: HLA, human leukocyte antigen; na, not available; Neg, negative; Pos, positive. In bold, 2A-TAV-reactive donors.