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PACT Update

Scale Up of Retroviral Transduction From 2-mL Wells to T75 Flasks

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Introduction

Retroviral vectors are commonly used for the genetic modification of clinical T-cells products. The transduction efficacy of primary T-cells can be enhanced dramatically by the use of retronectin to co-localize the vector with the T-cells (Murray et al, 1999). This requires centrifugation of retroviral vectors onto non-tissue culture-treated plastic surfaces, so that T-cells can be centrifuged onto the vector-coated retronectin.

In our Center we have used retronectin coated 24-well plates for transduction of CD3 and CD28 antibody activated T-cells (ATCs) or peptide activated virus-specific T-cells (VSTs), producing transduction efficiencies ranging from 60% to over 90% for ATCs and 40% to over 80% for VSTs (Lapteva et al, 2019). The use of 24-well plates has been feasible, because small numbers of T-cells can be transduced and then expanded to sufficient numbers for clinical use. Typically, 1.5 mL of retroviral vector per well is centrifuged for 1 hour at 2000 X G at room temperature onto retronectin-coated wells. The vector is then aspirated and immediately replaced with 2.5 – 5 x 10^5 T-cells in 2 mL of medium containing cytokines (IL-7 and IL-15), followed by a short low speed centrifugation and return to culture. Two days later the cells are transferred to G-Rex flasks for further expansion. Hence, starting with approximately 20 to 100 mL of peripheral blood, we transduce 5 to 36 x 10^6 ATCs on day 2-3 of culture and obtain sufficient transduced ATCs for cryopreservation between days 8 and 20 of culture, depending on the number of cells required for the clinical protocol.

However, if the titer of the retroviral is low, fewer cells can be transduced per well and some investigators demand minimal T-cell expansion to preserve products with an early differentiated phenotype. Hence greater cell numbers must be initiated requiring multiple 24-well plates for transduction. Although this has not led to contamination incidents, the manipulation of multiple 24-well plates introduces higher risks into product manufacturing and is not desirable from a good manufacturing practices standpoint. To scale up this procedure and shorten expansion times, we evaluated retroviral transduction in T75 flasks, evaluating both ATCs and VSTs using two different retroviral vectors and three donors per combination.

Material and Methods

Two clinical grade retroviral vectors, CAR.CD5.28z.CH3, lot TRV1702.B (CD5.CAR) and **TGF**βRII dcyt, lot TRV1804.C (**TGFβDNRII**) produced by the Vector Production Facility of the CAGT GMP (Houston, TX) were used to evaluate transduction of ATCs and VSTs, respectively. Standard transduction in 24-well plates was compared to transduction of varying cell numbers in T75 flasks. Cell counts were determined using a hemocytometer and trypan blue exclusion method, and transduction efficacies were analyzed by flow cytometry.

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Generation of retroviral vector-coated 24-well plates: Retronectin-coated 24-well plates or flasks (non-tissue culture-treated, Corning Costar, Corning, NY) were prepared by the addition of retronectin (RetroNectin, Takara Bio, Kusatsu, Japan) in Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco Grand Island, NY). After 4 to 6 hours at 37° C or between 16 hours and 3 days at +4 °C, 1 mL of retroviral vector per retronectin coated well or 20 mL of vector per coated flask was centrifuged for 1 hour at 2000 x G. Vector was aspirated prior to adding ATCs or VSTs.

Generation of CD5.CAR ATCs: ATCs were activated from cryopreserved peripheral blood mononuclear cells (PBMCs) from three different donors by culture on CD3/CD28 antibody (Miltenyi, Bergisch Gladbach, Germany) coated, non-tissue culture treated 24-well plates (Corning Costar, Corning, NY). Cytokines (10 ng per mL IL-7 and 5 ng per mL IL-15) were added on day +1 of culture. ATCs were transduced on day 3 at 0.375 x 10^6 cells per well or at 14 x 10^6 or 20 x 10^6 cells per flask by centrifugation onto the retroviral vector coated 24-well plates/flasks at 1000 x g for 10 minutes at room temperature. ATCs were fed with IL-7 and IL-15 (R&D Systems, Minneapolis, MN) as needed and transferred to G-Rex 10/100 flasks (Wilson Wolf, Minneapolis, MN) on day 4 of culture. Transduced ATCs were submitted for transgene expression analysis on day 7 and grown until day 9. Nontransduced ATCs were maintained in parallel as controls.

*Generation of **TGFβDNRII** transduced VSTs*. Human papillomavirus-specific T cells (HPVSTs) were generated from HPV-positive donors identified by the Papanicolaou test (Pap smear) (Ramos et al, 2013). HPVSTs received three cycles of stimulation. The first and second stimulations used mature dendritic cells (DCs) derived from CD14 positive PBMCs by magnetic bead selection (Miltenyi, Bergisch Gladbach, Germany) and culture in the presence of cytokines (IL-4, GM-CSF, IL-1β, IL-6, IL-12 and TNFα) (Leen et al, 2007). Mature DCs were pulsed with HPV-Pepmixes™ (overlapping peptide libraries representing the protein sequences of HPV16 E6 and E7 and HPV18 E6 and E7 (JPT Peptide Technologies, Berlin, Germany) and co-cultured with autologous CD14-negative PBMCs in the presence of IL-6, IL-7, IL-12 and IL-15. On day 9, the responder cells were re-stimulated with fresh pepmix-pulsed DCs and cultured in IL-7 and IL-15. Four days after the second stimulation, HPVSTs were transduced with the **TGFβDNRII** retroviral vector as described for the ATCs, with 1.5 mL of retroviral vector per well or 20 mL per T75 flask. HPVSTs were transduced at 0.5 x 10^6 cells per well (0.25 x 10^6 cells/cm^2) or in T75 flasks at10 x 10^6 cells/flask (0.13 x 10^6 cells/cm^2), 20 x 10^6 cells/flask (0.27 x 10^6 cells/cm^2) or 30 x 10^6 cells/flask (0.4 x 10^6 cells/cm^2 followed by centrifugation at 800 x g for 8 minutes at room temperature. Nontransduced control HPVSTs were maintained in parallel for each experiment. Three days after transduction, HPVSTs were stimulated for a third time with an irradiated antigen presenting complex comprising Pepmix™-pulsed autologous activated T-cells (ATC) and an HLA-negative costimulatory cell line (Ngo et al, 2014). Seven days after the third stimulation, HPVSTs were harvested and transduction efficiency and transgene copy number were determined.

NT		nontransduced control
Plate	0.5 x 10^6 cells per well of a 24 well plate	= 0.25 x 10^6 cells per cm^2
Plate9	0.375 x 10^6 cells per well of a 24 well plate	= 0.187 x 10^6 cells per cm^2
Flask10	10 x 10^6 cells per T75 tissue culture flask	= 0.13 x 10^6 cells per cm^2
Flask14	14 x 10^6 cells per T75 tissue culture flask	= 0.19 x 10^6 cells per cm^2
Flask 20	20 x 10^6 cells per T75 tissue culture flask	= 0.27 x 10^6 cells per cm^2
Flask30	30 x 10^6 cells per T75 tissue culture flask	= 0.4 x 10^6 cells per cm^2

Table 1: Nomenclature of samples for transduction at different conditions.

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Transgene and viability analysis. The CD5.CAR transgene expression on the cell surface was detected using goat anti-mouse anti-CH3 Fc-specific polyclonal antibody (Jackson ImmunoResearch, West Grove, PA). **TGFβDNRII** transgene expression was detected at the cell surface using the **TGF**β receptor II antibody (Abcam, Cambridge, UK) followed by PE rat anti-mouse IgG1 (BD Biosciences, San Jose, CA). Viability was determined using a hemocytometer and trypan blue exclusion method. Samples were acquired using a Gallios (Beckman Coulter,Brea, CA) or the Canto II, (BD Biosciences, San Jose, CA) instruments and analyzed with either FlowJo v.9 or Kaluza V2.1 software.

Integrated transgene copy number per cell. Genomic (g) DNA was isolated from approximately 3-5x10^6 transduced ATCs/HPVSTs cells using QIAamp DNA Blood Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. DNA concentration was determined by measuring the absorbance at OD260nm on NanoDrop 2000 spectrophotometer. Quantitative polymerase chain reaction (Q-PCR) was performed to determine the copy number of integrated retroviral transgene per single cell. Absolute quantification using the standard curve method was used. A standard curve was made using serial dilutions (3 to 300000 copies per reaction) of transgene encoding plasmid DNA with calculated copy number (based on plasmid length and weight). Q-PCR was performed with Custom TaqMan Gene Expression Assay (x20) containing primers and probe complementary to a specific sequence within transgene, and TaqMan Universal PCR Master Mix (x2) (all by Applied Biosystems, Life Technologies by Thermo Fisher Scientific, Grand Island, NY). Amplifications were performed on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Grand Island, NY) according to the manufacturer's instructions. Briefly, each PCR included 25 µL of total reaction volume with 900 nM of each primer, 250 nM of the probe, and 100 ng of DNA template. Transgene copy number per cell was calculated based on estimation that 1000 ng of human genomic DNA contains 150,000 diploid genomes.

Statistical analysis. The data was analyzed using Prism GraphPad software by one way ANOVA with Tukey's post-hoc test. P value of below 0.05 was considered as statistically significant.

Results & Discussion

CAR.CD5.28z.CH3 transduced ATCs

Viability

The viability of transduced ATCs was determined on day 4 post transduction. Results are summarized in **Table 2** and **Figure 1**.

	Donor 1		Donor 2		Donor 3		Average	
	Viability [%]	TE [%]	Viability [%]	TE [%]	Viability [%]	TE [%]	Viability [%]	TE [%]
non transduced	94.5	1.48	87.0	1.24	93.5	1.76	**91.7**	**1.5**
24 well plate (0.375 x 10^6 cells/well)	95.9	98.8	83.8	98.4	91.6	86.5	**90.4**	**94.6**
T75 flask (14 x 10^6)	95.2	98.4	90.9	98.7	92.5	88.1	**92.9**	**95.1**
T75 flask (20 x 10^6)	92.4	98.7	94.8	99.0	92.8	88.5	**93.3**	**95.4**

Table 2: Viability (Trypan Blue) and transduction efficacy (TE) of CD5.CAR transduced ATCs. *ATCs transduced in 24-well plates and flasks and nontransduced ATCs were counted on day 4 post transduction in three donors.*

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Transduced ATCs met viability release criterion of >70% as they were >90% viable cells in all tested conditions. Viability for cells grown in T75 flasks was slightly higher (however, not statistically significantly different p=0.56) than for cells grown in 24-well plates and viability was not affected by transduction.

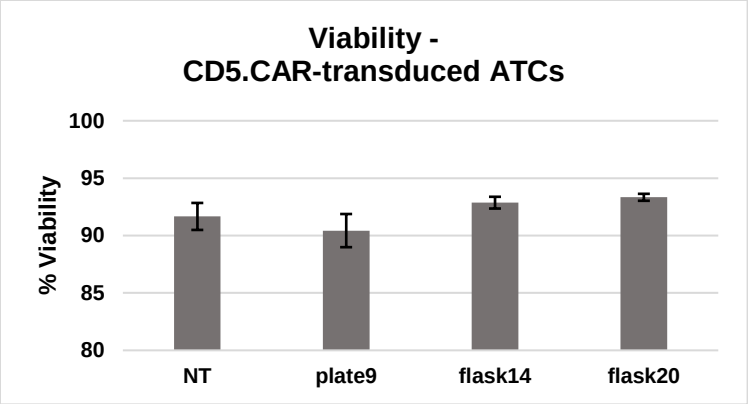

Figure 1: Viability of CD5.CAR-transduced ATCs. *ATCs were harvested from wells and T75 flasks on day 4 post transduction. Viability was determined by the trypan blue exclusion method.*

Transduction Efficacy

Samples for transgene expression were acquired on day 4 post transduction for all three donor products. Results are summarized in **Table 2** and **Figure 2**.

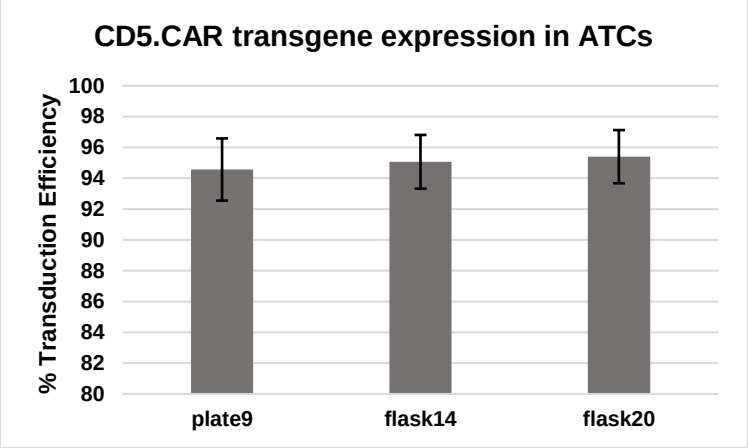

Figure 2: CD5.CAR transgene expression in ATCs *transduced in 24-well plates and in T75 flasks were harvested on day 4 post transduction and submitted to flow cytometry for analysis.*

With an average CD5.CAR transduction efficacy of >94%, transduction efficacies were comparable between all three conditions and exceeded the protocol specific release criterion of 40%. There was no statistical significant difference (p=0.35) for cells transduced in plates or flasks.

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MFI and Vector Copy Numbers

The mean fluorescence intensity (MFI) of the transgene expression was determined on day 4 post-transduction. The MFI was similar for all tested transduction conditions in all three donors. One representative donor is illustrated in **Figure 3**.

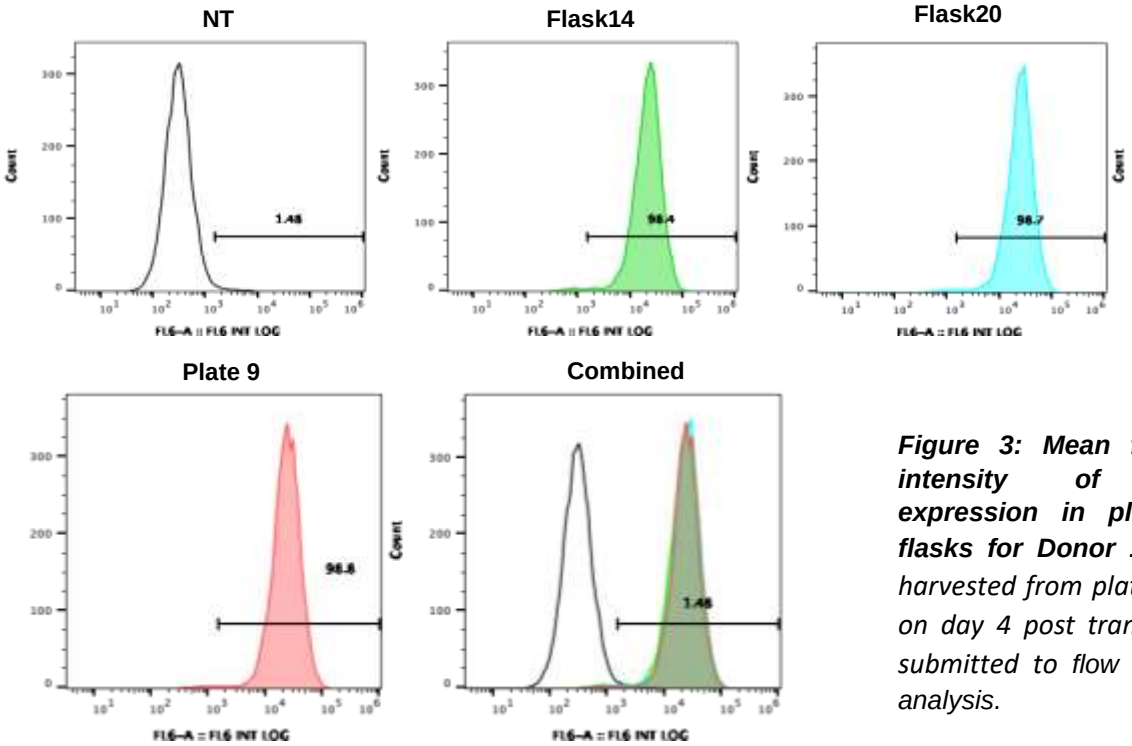

Figure 3: Mean fluorescence intensity of transgene expression in plates versus flasks for Donor 1*. ATCs were harvested from plates and flasks on day 4 post transduction and submitted to flow cytometry for analysis.*

Expansion Rates

Cells were counted on days 1, 4 and 6 post transduction and to total fold expansion rate CD5.CAR transduced ATCs transduced in plates and flasks were compared. There were no statistically significant differences (p=0.36) in the fold expansion between tested conditions (**Table 3** and **Figure 4**), although there was a trend towards greater proliferation in flasks.

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	# cells x10^6									Average cumulative x-fold change		
	Donor 1			Donor 2			Donor 3					
	plate 9	flask 14	flask 20	plate 9	flask 14	flask 20	plate 9	flask 14	flask 20	plate 9	flask 14	flask 20
transduced (Day 0)	9	14	20	3.3	14	20	9	14	20	NA	NA	NA
Day 1 PT	25.5	17.5	18	2.7	10.6	19.5	7.2	10	15.8	1.5	0.9	0.9
Day 4 PT	139.2	166	122	9.3	126.6	154	42.6	103.2	196	7.2	9.9	9.3
Day 6 PT	621.2	780	657	44.8	455.6	796.4	114.4	204	448	28.9	33.9	40.1

Table 3: Cell counts and average cumulative fold change of CD5.CAR transduced ATCs in plates and flasks. *Cells were counted on days 1, 4 and 6 post-transduction (PT).*

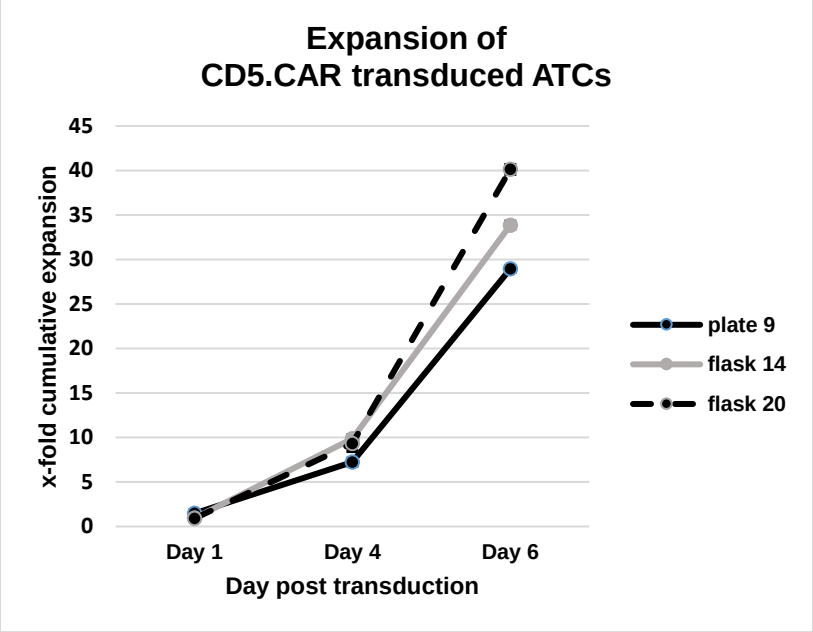

Figure 4: Fold cumulative expansion of CD5.CAR ATCs transduced in plates versus flasks. *Cells were counted on days 1, 2 and 6 after transduction.*

TGFβDNRII transduced HPVSTs

HPVSTs were harvested seven days after the third stimulation for determination of transduction efficiency (TE) and vector copy number (VCN) per transduced cell. There was no statistically significant difference in transgene expression (p=0.26) or VCN (p=0.74) between cells transduced in plates and flasks at 10 x 10^6 cells/flask and 30 x 10^6 cells/flask respectively (**Table 4** and **Figures 5** and **6)**. HPVST proliferation was also similar (not shown).

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	Donor A		Donor C		Donor F23		Average	
	TE [%]	VCN	TE [%]	VCN	TE [%]	VCN	TE [%]	VCN
24 well plate (0.5 x 10^6 cells/well)	20.8	3.2	41.4	1.2	48.8	2.9	**37.0**	**2.4**
T75 flask (10 x 10^6)	20.0	1.7	29.7	1.3	42	4.7	**30.6**	**2.6**
T75 flask (20 x 10^6)	14.3	2.4	48.5	0.4	ND	ND	**31.4**	**1.4**
T75 flask (30 x 10^6)	18.0	1.9	22.0	1.9	46.1	2.1	**28.7**	**2.0**

Table 4: Effect of transduction vessel on transduction efficacy and vector copy number*. The transduction efficiency (TE) and vector copy number (VCN) for HPVSTs from 3 different donors transduced with the* ***TGFβDNRII*** *vector in 24 well plates and T75 flasks is shown on day 7 post stimulation #3. TE and VCN were not analyzed in the "Flask 20" (i.e. 20 x 10^6 HPVSTs seeded in a T75 flask) for donor F23, therefore the data for this condition was not statistically analyzed).*

Transduction Efficacy

There was no statistically significant difference in transgene expression (p=0.26) for HPVSTs in plates and flasks at 10 x 10^6 cells/flask and 30 x 10^6 cells/flask, respectively (**Figure 5**). The release criterion of 20% transduction was achieved for all donor HPVSTs transduced in flasks.

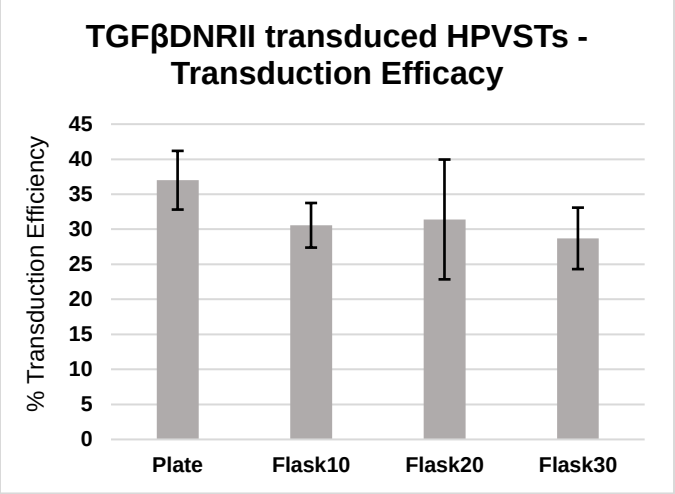

Figure 5: HPVSTs could be transduced with the TGFβDNRII retroviral vector in T75 flasks. *Shown is transgene expression on day 7 after the third stimulation.*

Vector Copy Number

There was no statistically significant difference in **TGFβDNRII** transgene copy number in HPVSTs (p=0.74) between cells transduced in plates and flasks at 10 x 10^6 cells/flask or 30 x 10^6 cells/flask, respectively. In all cases, the vector copy number was below 5 copies per cell, which is the FDA recommended maximum (see **Figure 6**).

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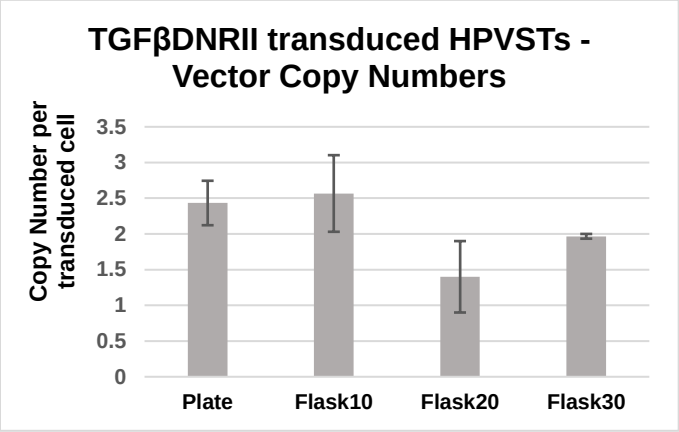

Figure 6: Vector copy number comparison between HPVSTs transduced in wells and flasks in. *HPVSTs transduced in wells and T75 flasks were harvested 7 days post stim #3 and submitted for transgene analysis. The copy # per cell was calculated based on the percent of transduced cells as measured by flow*

	ATCs			HPVSTs			
	2 cm² well	75 cm² flask		2 cm² well	75 cm² flask		
Cells per well/flask x 10^6	0.375	14	20	0.5	10	20	30
Cells per cm² x 10^6	0.187	0.19	0.27	0.25	0.13	0.27	0.4
% transduced cells	95	95	95	37	31	31	29
Vector copy # per cell	ND	ND	ND	2.4	2.6	1.4	2.0

Summary Table 5. *Relationship between cell density per unit of surface area at transduction, transduction efficiency and vector copy number per cell*

Conclusions

ATCs can be successfully transduced with the CD5.CAR in non-tissue culture treated T75 flasks, meeting release criteria in all conditions. Viability, transgene expression and MFI were similar for all transduction conditions. The seeding densities tested in flasks did not negatively impact any of the analyzed parameters, and higher cell numbers increased the rate of CD5.CAR ATC expansion. Consequently, moving the transduction procedure from 24-well plates into T75 flasks simplifies the otherwise complex and potentially risky open-system process of seeding and harvesting T-cells from multiple plates. Further, the use of higher numbers of ATCs as starting material combined with a trend towards better expansion rates allow generation of high cell numbers required for clinical protocols.

TGFβDNRII transduced HPVSTs could also be transduced in flasks, but there was variability between donors, suggesting that more donors should be tested to identify the optimal flask transduction condition for the **TGF**βDNRII vector.

We are beginning to implement transduction in flasks in our GMP facility for certain vectors, but the optimal cell numbers used will need to be established for each vector and cell type combination.

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