Towards a surrogate system to express human lipid binding TCRs

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Abstract:

What gives a protein the ability to sensitise, to be an allergen, in particular a food allergen, is not well understood. Previously we reported that natural nut lipids were necessary for sensitization and that Natural Killer T Cells (NKTs) must play a critical role in the development of Brazil nut –allergic responses.

A major bottleneck in further understanding the interaction of nut natural lipids with the cells of the human immune system is the lack of well-characterized lipid responsive human cell lines. In the present study, we engineered human T-cell receptor (TCR) sequences TRAV10 (clone J3N.5T) and TRBV25 (clone BM2a.t) responsive to α-GalCer into a stable murine iNKT hybridoma cell line. This system has shown to be problematic as far as expression of new and functional human TCR sequences is concerned. To overcome this limitation, we then show that the expression of human TCR sequences has been achieved using a bidirectional promoter on a plasmids or a lentivirus system. This system employed murine or human stable DC cell lines as lipid presenting cells and a stable T cell line as a surrogate system. Further, we show that the commercial human Jurkat cell line containing an inducible secreted luciferase reporter construct regulated by human NFAT binding sites was functional and can be used for a transient expression of human TCRs in a lipid screening program. We also show that transfection efficiencies were improved using the lentivirus polycistronic constructs containing the P2A sequence in a TCR $\alpha\beta/\gamma\delta$ null cell line (Jurkat 76). These results suggest that the mis-pairing of the endogenous α/β TCR during ER folding in the presence of the new human TCR sequences could be impairing the functionality of the TCR lipid receptors. This will help towards a surrogate system to express functional human lipid binding TCR sequences. These are important first steps in the establishment of human cell-specific lipid responsive libraries for the study of natural lipid substances.

Introduction

What makes a protein an allergen with respect to the ability to sensitise and, in particular a food allergen, is not well understood. Previously Kean et al reported that SFA8, the 2S albumin from sunflower, and not the major nut allergen Ber e 1 was able to polarize dendritic and T helper (Th) cell responses in mice with production of IL-12 p40 and TNF-alpha. Transcription analysis showed increased Th1 transcrition factor T-bet with respect to both proteins, but some Th2 GATA-3 with respect to Ber e 1 (Kean et al., 2006). Subsequently, it was shown that Brazil nut lipids were required for sensitization with Ber e 1 (Dearman et al., 2007) and that one particular complex lipid fraction (lipid C) was able to induce specific Ber e 1 anaphylactic antibodies in naïve animals. Subsequently, in kinetic experiments we have also shown that Ber e 1 can indeed accommodate one lipid molecule (stoichiometry 1:1) with K_d of 5.6±0.1 µM (Mirotti et al., 2013; Rundqvist et al., 2012). The requirement for natural lipids from nuts as a critical component for the intrinsic allergenicity of Ber e 1 was further demonstrated when human T-cell lines derived from nut allergic patients were shown to produce IL-4 to Ber/lipid C in a CD1d dose dependent manner. Ja18 and CD1 knock out experiments further implicated Natural Killer T Cells (NKTs) in the response (Mirotti, Florsheim et al. 2013). Altogether, these results illustrate the essential role of the natural lipid fraction in nut protein sensitization and strongly suggest that NKTs play a critical role in the development of Brazil nut –allergic response (Mirotti et al., 2013).

NKT cells are unique lymphocyte subpopulations characterized by co-expression of surface markers from conventional NK and T cells. NKTs make up 0.01-2 % of human peripheral blood mononuclear cells and have been shown to be important in all aspects of immunity such as development, regulation of autoimmune, allergic, infectious and neoplastic responses (Godfrey et al., 2015; O'Konek et al., 2012; Taniguchi et al., 2003). In response to T-cell receptor (TCR) engagement NKTs rapidly produce cytokines involved in the activation of dendritic cells (DCs), NK cells, macrophages, B cells and conventional T cells amongst others. NKTs, via TCR engagement, can recognize lipid antigens that are presented by the nonclassical MHC I-like CD1 receptors expressed on the surface of antigen presenting cells (APCs), which is significantly different from T cells (de Jong, 2015). Lipid antigens presented

via CD1 generally respond to changes in extracellular environment (Dowds et al., 2014). Within the context of allergies, NKT cells were shown to participate in Th2 responses through a CD1d-dependent mechanism (Mirotti et al., 2013).

One of the major bottlenecks in the studies of the interaction of natural lipids with the cells of the human immune system is the lack of human lipid responsive cell lines. In the present study, we attempted to engineer human TCRs into a stable murine hybridoma cell line employing well described synthetic TCR sequences and utilizing the widely used marine sponge glycolipid α-galactosylceramide (α-GalCer) as an activator (Bai et al., 2012). Further, our work with murine and human stable DC cell lines has taken the work to commercial surrogate human T cells (Jurkats). The transient expression in several Jurkat backgrounds via plasmid or via lentivirus was also analyzed. Thus, the methodology, results and discussion presented herein will help towards a cell system able to express human responsive lipid binding TCR sequences. These are important first steps in the establishment of cell specific lipid responsive libraries for the study of natural lipid substances.

1. Material and methods

1.1 Cell lines

JAWSII a mouse dendritic cell line was purchased from the American Tissue culture collection (ATCC) (#CRL-11904). DN32.D3 a mouse double negative iNKT hybridoma cell line was a gift from Professor Albert Bendelac, Howard Hughes Medical Institute, University of Chicago, USA. Jurkat Lucia cells (human T cell containing NFAT Luciferase reporter gene) were purchased from InvivoGen. MUTZ3 cells (human DC cell line) and ACC 35 cells (human urinary bladder carcinoma) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany (# 295 and 5637 respectively). MUTZ has been reported to express CD1a, CD1b, CD1c and CD1d (Masterson et al., 2002). 293T cell, a human embryonic Kidney cell, was purchased from the American Tissue culture collection (ATCC) (#CRL-3216). Jurkat 76 is a human TCR $\alpha\beta/\gamma\delta$ null cell line kindly donated by M. Heemskerk (Heemskerk et al., 2003) that has been reported as a stable recipient for new TCR sequences (Guo et al., 2016).

1.2 Chemicals

RPMI 1640 media, heat inactivated FBS, opti-MEM, DMEM and α-MEM were purchased from Gibco, UK. Zeocin and blasticidin were purchased from Thermo Fisher Scientific, UK. α-Galactosyl ceramide-KRN7000L was obtained from Tebu-bio Ltd, UK. Murine GM-CSF was purchased from PeproTech, UK. L-α-Lyso phosphatidylethanolamine;, HEPES, Beta-Mercaptoethanol, Histopaque, Puromycin, Polyethylenimine, TNF-α, Ionomycin and concanavalin A were purchased from Sigma UK. Sulphatide and glucocerebroside were from Matreya LLC, USA. L-α-Lysophosphatidyl inositol sodium salt, phosphatidic acid and β-sitosterol were from Fluka, UK. Phorbol 12-myristate was from Invivogen. 3-sn-Phosphatidyl coline from soya bean from BDH, UK. Fugene 6 Transfection Reagent was bought from Promega, UK. Sodium pyruvate and L-Glutamine were purchased from Gibco. Polybrene was purchased from Millipore Co EMD. Luciferase substrate QUANTI-Luc was bought from InvivoGen.

1.3 Antibodies

Anti-Vα24PE antibodies were from Beckman Coulter. eBioscience Human IL-2 ELISA Ready-Set-Go Kit was bought from Fisher Scientific.

1.4 pMJA219 TRBV25/TRAV10 expression plasmid

A synthetic expression DNA plasmid (pMJA219, GenBank MH782476) containing TCR receptors has been synthesised (Geneart) based on the backbone of the mammalian expression plasmid pcDNA3.1(+)(Invitrogen). The α/β TCR sequences used in pMJA219 design have been previously described as α-GalCer specific using lipid loaded tetramers (Brigl et al., 2006). pMJA219 is driven by the bidirectional Cytomegalovirus promoter from pBI-CMV1 (Clontech) and flanked by the rabbit β-globin and bGH (bovine Growth Hormone) polyadenylation sequences at the 3' end of TRBV25 and TRAV10 sequences respectively. The complete TCR expression plasmid contains the human α-GalCer responsive TRAV10 (clone J3N.5T, GenBank DQ341448.1) and TRBV25 (clone BM2a.t, GenBank DQ341454.1) cDNA complete sequences without introns (Brigl et al., 2006). pMJA219 carries selectable markers for Ampicillin for E. coli and G418 (Geneticin) for mammalian cells.

1.5 Lentivirus expression

The lentivirus expression was carried out using the 3 plasmid second generation system essentially as described by Roth et al. (Roth et al., 2017). The psPAX2 (packaging) and pMD2.G (VSV-G expressing envelope) were gifts from Didier Trono (Addgene plasmid 12260 and 12259 respectively). pSin-EF2-Nanog-Pur (target vector) was a gift from James Thomson (Addgene plasmid 16578). Two target vectors were assembled. pMJA285 (Genbank MH782473) contained the bidirectional CMV promoter driving TRAV10 (GenBank DQ341448.1) and TRBV25 (Genbank DQ341454.1) sequences in opposite orientation as described for the pMJA219, XbaI and SpeI fragment were inserted into pSin-EF2-Nanog-Pur. pMJA289 (GenBank MH782475) was assembled by PCR overlapping primers and contained the same TRAV10/TRBV25 constructs in a dicistronic orientation driven by EF2 promoter and the α/β TCR sequences separated by the GSG-2A self-cleavage peptide sequence GSGATNFSLLKQAGDVEDNPGP (Liu et al., 2017). For the transduction, the target cells (Jurkat Lucia or Jurkat 76) were counted. For each transduction, 105 cells were used. The cell suspension was centrifuged (1000 rpm, 5 min) in separated tubes and the supernatant was discarded. Cell pellets were resuspended in 2 ml virus supernatant. Polybrene was added at a final concentration of 8 μg/ml. The suspension was transferred into a 6 well plate. The plate was sealed with parafilm centrifuged for 90 min at 1200 rpm (spin transduction). Afterwards, the cells were carefully resuspended and incubated for another 6 hours at 37 °C. Subsequently, the cells were centrifuged and resuspended in fresh Jurkat media. The supernatant was carefully discarded and inactivated. 48 hours post transfection, the cells were split. Puromycin antibiotic was added to one well at a final concentration of 0.5 μg/ml. One week later, the cells were tested in a co-culture. Furthermore, the cells were stained with Anti-Vα24PE antibodies and analysed in flow cytometry as described below.

1.6 Co-culture

DC-lipid load: human DC MUTZ-3 were cultured at 37°C, 5 % CO_2 in media containing α-MEM with FBS (20%), condition media (5637 cell line supernatant at 20%) and human GM-CSF (5ng/ml) according to cell culture instructions (DSMZ). Mouse DC JAWSII were cultured in the same conditions but in α-MEM with FBS (20%), supplemented with L-glutamine (4mM) Na-pyruvate (1mM) and murine GM-CSF (5ng/ml). For lipid loading

MUTZ-3 or JAWII were counted, centrifuged and resuspended in fresh DC media (RPMI 1640, 10% FBS, 1% PenStrep and 5% DMSO) to a final density of $2.5x10^4$ cells/30μl and added onto each well of a 96 well plate. Lipid or α-GalCer was added to MUTZ or JAWSII cells to a total 50 μl/well reaction mixture and incubated for 24h at 37°C, 5 % CO_2.

T cell transfection: Jurkat Lucia cells were cultured in Jurkat media (RPMI 1640, 10% FCS and 100 μg/ml Zeocin) accordingly to the manufacturer's instructions (InvivoGen). The media was changed every three days, by switching between media with and without Zeocin. Jurkat 76 cells were cultured in Jurkat76 media (RPMI 1640, 10% FCS, 1% L-Glut, 1% sodium pyruvate, 1% PenStrep). For transfection, the cells were counted, centrifuged and resuspended in 100 μl/well fresh Jurkat media to a final density of $2x10^5$ cells/well and dispensed onto a 96 well plate. The transfections were performed by the procedure described in the Fugene 6 Transfection Reagent protocol (Promega,UK). Briefly, a total of 50 μl of transfection reaction was prepared in RPMI media: 3μl Fugene 6 reagent was added followed by 1μg plasmid DNA and incubated for 30 min RT. Finally, all transfection reactions were added drop-wise to each well containing Jurkat cells and incubated for 24h at 37°C, 5 % CO_2.

Co-transfection and readout: After 24h the lipid loaded MUTZ3 cells were mixed and added (50μl/well) to transfected Jurkat Lucia cells onto a 200μl final reaction containing $2.4x10^4$ DC+ $2x10^5$ T cells) and incubated overnight at 37°C, 5 % CO_2. PMA (50ng/ml) and ionomycin (3μg/ml) were used as positive controls to stimulate T cells. After 24h incubation, the supernatant was harvested, and the secreted luciferase activity measured by QUANTI-Luc assay containing coelenterazine substrate. For this, 20μl of each sample was transferred onto Optiplate 96 plate, 50μl/well QUANTI-Luc added and immediately measured onto a luminometer (Turner Biosystems) and analysed in MS-Excel. The remaining supernatant was stored at -20ºC or used in an IL-2 Sandwich ELISA determination (384 well plate) using HRP and TMB as substrate following the Ready-Set-Go! Kit according to the manufacturer's instructions (Fisher Scientific). The results were read at 450 nm using Tecan Infinite M200 PRO plate reader, data collected by Magellan software and transferred to a MS-Excel spreadsheet for analysis.

1.7 Flow cytometric analysis

Firstly, cells in each well were harvested into each FACS tubes, 2 ml of PBA (500 ml PBS containing 30% BSA and 20% Sodium Azide) was added and centrifuged for 5 min at

300g. Supernatants were discarded, and cell pellet was re-suspended. 5μl antibodies (anti human Vα24 antibody) were added and incubated at 4°C for 30 min in the dark. Afterwards, 2 ml of PBA was added to each tube. Supernatants were discarded, pellet re-suspended and cells were fixed in 0.5 ml PBA. Analysis was carried out on the Flow Beckman Coulter flow cytometer FC500 (Beckman Coulter life Sciences, USA), at the Flow Cytometry Facility (University of Nottingham). After quantification by FC500 the data was analysed using Kaluza software (Beckman Coulter).

2. Results and discussion

2.1 Lipid responsive TCR, murine system

The murine hybridoma cell line DN32.D3, originally described by A. Bendelac (Lantz and Bendelac, 1994), has been widely used by many groups working with lipid binding and NKT cell activation. The TCR of DN32.D3 cell line consists of Vα14 Jα18 paired with Vβ8, Vβ7 or Vβ2 and the cell line expresses the NK1.1 marker, an identifier of NK cells. The characteristics of DN32.D3 cell line have been described in great detail elsewhere (Jordan-Williams et al., 2013; Kim et al., 2010, 2006). This double negative hybridoma murine cell line when presented in co-culture to the murine DC cell line JawsII has shown a sigmoidal IL-2 dose response curve with increasing α-GalCer concentration (Figure 1A). This response is blocked by murine anti-CD1d antibodies, hence CD1d specific, and is not further amplified by murine anti-CD3/CD28 beads. Furthermore, the response seemed to be IL-2 specific as only the IL-2 cytokine was detected, however,a Luminex panel containing only 6 cytokines (IL-2, 4, 5, 10, 12 and IFN-γ) was used (results not shown).

As expected, the dose response curve of DN32.D3 against α-GalCer in co-culture is species-dependent. DN32.D3 showed a higher sensitivity to lower levels of α-GalCer when the glycolipid is presented by the murine JAWSII than when presented by the human MUTZ3 DC cell lines (Figure 1B). Regarding lipid specificity, DN32.D3 has shown to be quantitatively specific to α-GalCer in the murine JAWSII system (Ghumra and Alcocer, 2017) with a high background response for the other lipids tested (Figure 1A). The human

DC system MUTZ3, although less sensitive, responded in addition to α-GalCer to LPE and SDS (Figure 1B).

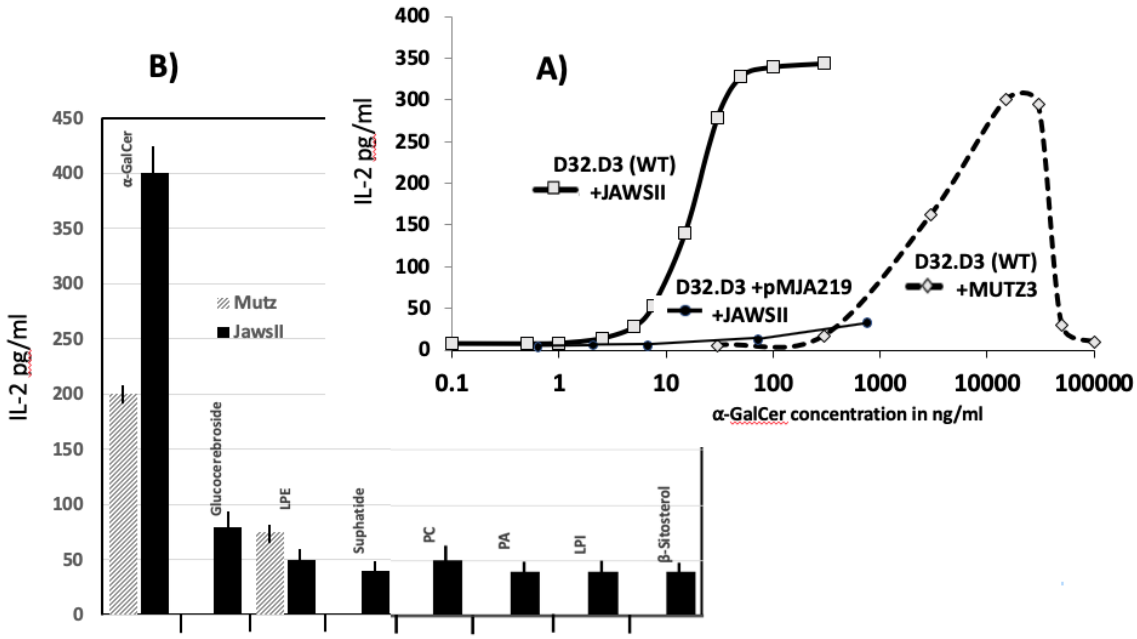


Figure 1: α-GalCer dose response of DN32.D3 co-cultured with JAWSII or MUTZ3 cell lines. *A) IL-2-α-GalCer dose response of DN32D3 wild type and transfected DN32.D3 with the plasmid pMJA219 with JAWSII or MUTZ3 as indicated after 72h 37°C. B) IL-2-lipid specificity response for DN23.D3 and MUTZ3 or JAWSII, lipids at 1ug/ml for JAWSII and 10ug/ml for MUTZ3. LPE: lyso-Phosphatidyl ethanolamine, PC: phosphatidyl choline, PA: Phosphatidic acid, LPI: lyso-phosphatidyl innositol. All co-cultures were performed at density of 5x10^5 cells/well of DN32.D3 and 5x10^4 cells/well of APC.*

In an attempt to engineer new functions and humanise DN32.D3, a plasmid (pMJA219) containing the α-GalCer responsive human TRAV10 and TRBV25 sequences was constructed (Fig 2). The α/β TCR sequences used in pMJA219 design have been previously described as α-GalCer specific using lipid loaded tetramers (Brigl et al., 2006). In order to achieve TCR α/β equimolar expression a mammalian pMJA219 plasmid was designed that contained the bidirectional CMV promoter driving the TCR sequences. After transfection and selection for 3 weeks on Gentamicin, FACS analysis using TRAV10 specific human antibodies (V24α sequence) confirmed that 60-80% of pMJA219 DN32.D3 transfected cells have displayed the specific human TRAV10 and none on the controls. These results confirmed that the bidirectional promoter is functional and that the murine cell line DN32.D3 can express a human α chain. However, when the pMJA219 transfected DN32.D3 cells were co-cultured with the murine DC JawsII they did not show any

improvement on their sensitivity to detect α-GalCer as shown in Figure 1A or to respond to PMA/ionomycin (not shown). These transfected cells were also irresponsive when co-cultured with the human MUTZ3 system.

Whether these results were a product of competition with the murine CD3 as suggested by some authors (Ahmadi et al., 2011) or by mis-pairing with the endogenous murine α/β TCR during ER folding (Knies et al., 2016; Sommermeyer et al., 2006) remains to be clearly demonstrated. What is clear from this exercise is that the over expression of the human α/β chains clearly disrupted the function of the endogenous murine chains (Fig 1A).

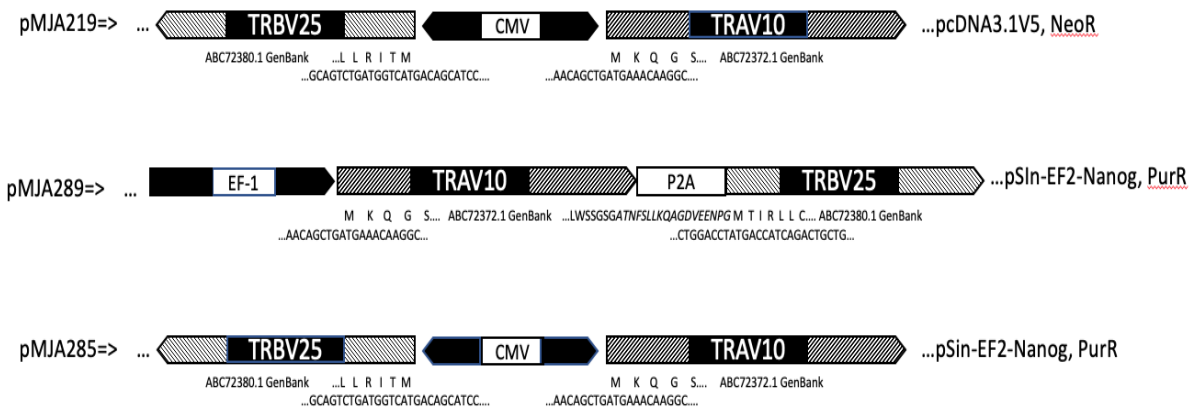


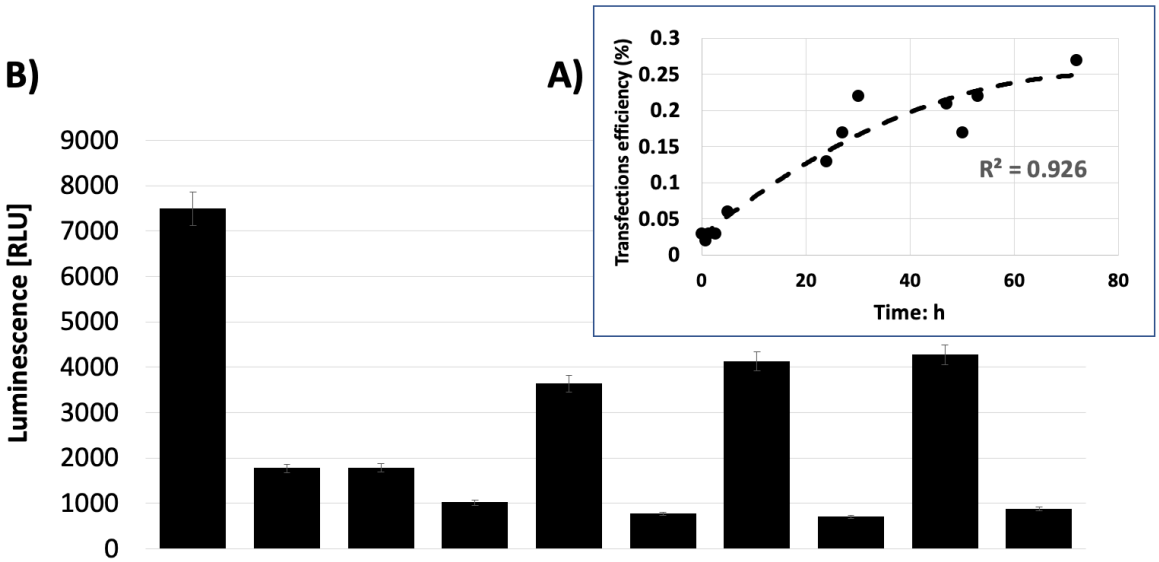
Figure 2: ***Diagrammatic TCR clones designed and constructed for this work****. pMJA219 is a mammalian NeoR plasmid (GenBank MH782476) driven by a bi-directional CMV promoter (pBI-CMV1, Clontech). pMJA285 (GenBank MH782473) is a similar construct but cloned into the lentivirus pSin-EF2-Nanog, PurR background. pMJA289 is a polycistronic construct into which the P2A cleavage sequence has been engineered. The AA proteolytic cleavage sequence of the P2A is indicated in italic. pMJA288 is pSin-EF2-Nanog lentivirus vector used in the cloning but without any insert.*

2.2 Jurkat Lucia cells

Attempts to disrupt the wild type TCR murine chains from DN32.D3 before the human TCR transfection were made by CRISPR, but the resulting clones did not show any murine α/β expression (data not shown). None of the DN32.D3 knockout cells produced significant amounts of IL-2, or responded to PMI/ionomycin, or were affected by the presence of the human TRAV10/TRBV25 expression construct. This work therefore is not shown here. It seems that due to its hybridoma nature and after a large number of passages, DN32.D3 has suffered a great number of recombinations plus cumulative

mutations that have resulted in an almost unusable cell line as far as expression of new TCRs is concerned.

A human stable cell line Jurkat Lucia was used instead of the murine DN32.D3. This cell line contains an inducible secreted coelenterazine luciferase reporter constructs regulated by human NFAT binding sites. No data is currently available on TCR or indeed CD3 expression of this particular cell line. pMJA219 was transfected into Jurkat Lucia and TRAV10 expression was measured by FACS in a time course experiment. (Figure 3A). Poor transfection (a maximum of only 0.3 %) was observed but further analyses were nevertheless carried out. The TRAV10/TRBV25 transfected Jurkat Lucia showed higher levels of luminescence compared to non-transfected cells when co-cultured with the human dendritic cell MUTZ3 loaded with α-GalCer (Fig 3B). Whether the APC, in this case MUTZ3, had time and the right conditions to achieve the optimal maturity was tested by incubation in three different maturation/conditioning media (M1, M2 and M3) as shown in Figure 3B. As reported these media contained different amounts of TNF-α, IL-4 hGM-CFS and long and short incubations (Masterson et al., 2002; Ning et al., 2011). From these findings M1 gave the highest signal and therefore was used throughout the remainder of the study.



M1	M1	M1	M1	M2	M2	M3	M3	-	-
219 DNA	-	219 DNA	-	219 DNA	-	219 DNA	-	-	-
α-GalCer	α-GalCer	-	-	α-GalCer	α-GalCer	α-GalCer	α-GalCer	-	-
-	-	-	-	-	-	-	-	I+P	-

Figure 3: ***Luminescence of co-culture human DC Mutz3 + Jurkat Lucia T cells****. A) Time course experiment showing the stability of the transfected TCR genes encoded by pMJA219 in Jurkat Lucia T cells as measure by V24α by FACS. B) Luminescence readings of triplicate wells after 24h of co-incubation of MUTZ3 and Jurkat Lucia cells at density of 2x10^5 each/well at 37°C, 5 % CO_2. M1-3 refers to the maturation treatment that Mutz has undergone. M1=lipid+24h in RPMI 1640, 10% FBS, 1% PenStrep and 5% DMSO. M2=Lipid+24h in α-MEM 60%, 20%FBS, 100ng/ml hGM-CSF, 10ng/ml IL-4, 75ng/ml TNF-α (Ning et al., 2011). M3=lipid+24h in alpha-MEM 60%, 20%FBS, 50ng/ml hGM-CSF, 20ng/ml IL-4, 12ng/ml TNF-α* (Masterson et al., 2002)*. I+P=Ionomycin+PMA.*

These results have demonstrated that the synthetic human αβ sequences present in the plasmid pMJA219 were functional, responded to α-GalCer and corroborated previous work by Brigl, van den Elzen et al. (Brigl et al., 2006). These data suggested that the commercial human Jurkat Lucia cells could be used for a transient expression of human TCRs in a lipid screening program. The low transfection rate and stability of these cells is questionable and warrants the need to further this line of investigation. Whether, as reported (Guo et al., 2016), an over expression of CD3 could bypass the Jurkat's known TCR expression bottle neck and improve the functionality of the cell line, remains to be shown.

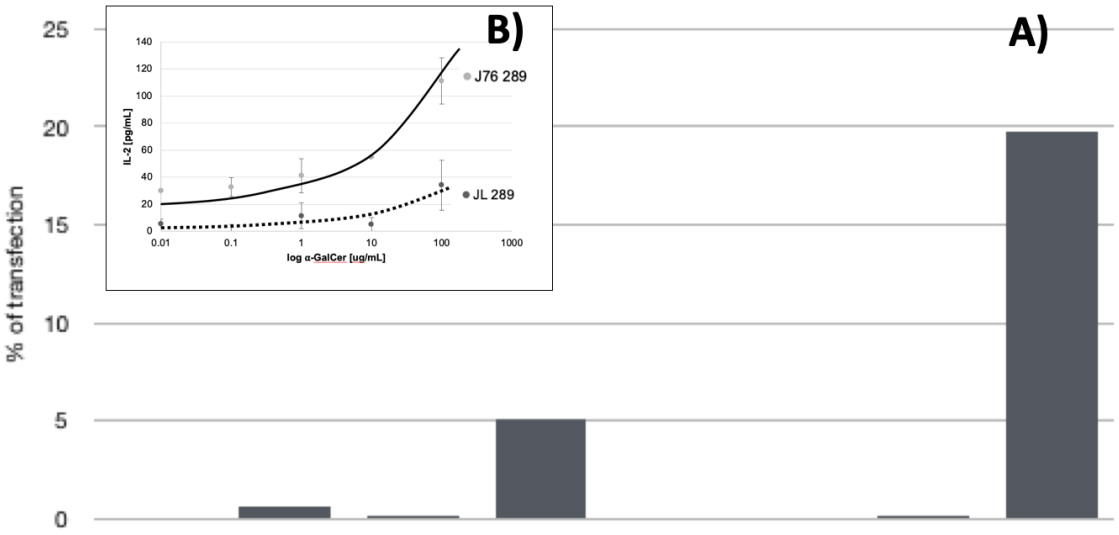
2.3 Lentivirus system

One alternative for plasmid driven TCR expression in T lymphocytes is the use of a lentivirus expression system as previously reported (Zhou and Buchholz, 2013). In this 3 plasmid system the packaging and envelope genes are mixed with the target or transfer plasmid (3:1:3) and the virus packed in receptor cells. For this, the target plasmid pMJA285 has been designed and constructed that contained the α/β TRAV10/TRBV25 sequences and similarly to pMJA219, was driven by the CMV bidirectional promoter (Figure 2). In another strategy the target plasmid pMJA289 (Figure 2) was produced containing the α/β genes in a discistronic configuration separated by the GSG-2A self-cleavage sequence, as described by Liu et al. (2017). When these viruses containing α/β TCR sequences were transfected into the Jurkat Lucia background, the dicistronic construct showed a higher transfection rate than the CMV construct after 72h, as monitored by FACS using anti human Vα24 Ab (Figure 4A). The human TCR expression achieved with the protease cleavage construct containing the P2A sequence (pMJA289)

was higher than the CMV bidirectional construct. These results are in agreement with Thomas et al., (2010) who stated that P2A improves the equimolar expression of both genes as well as leads to higher levels of cell-surface TCRs.

Interestingly, much higher transfection efficiency with the same virus constructs was obtained with the cell line Jurkat76 when compared with Jurkat Lucia (Figure 4), suggesting a more efficient assembling of the TCR/CD3 complex in this cell line. Jurkat 76 is a human TCR αβ/γδ null cell kindly donated by M. Heemskerk (Heemskerk et al., 2003) that has been reported as good and stable recipient for new TCR sequences (Guo et al., 2016). Thus, the results presented here corroborated the findings from both groups and suggests that indeed the mispairing of the endogenous α/β TCR during ER folding in the presence of the new human TCR sequences impaired the functionality of the lipid receptors.

Despite the high transfection efficiency, not all the expressed TCRs seemed to be functional. IL-2 and luciferase release after co-culture with α-GalCer were not much increased when compared to the transient plasmid expression of pMJA219, although the expression of the new human αβ sequences in the Jurkat 76 background produced reliable titration curves (Figure 4B) and reduced the time for obtaining stable lipid responsive transfected Jurkat cells. These results ratified the results in Figure 4A and show a higher response to α-GalCer of J76 than JL when transduced with plasmid pMJA289.



JL	JL+285	JL+288	JL+289	J76	J76+285	J76+288	J76+289
-	CMV	empty virus	GSG-2A	-	CMV	empty virus	GSG-2A

Figure 4: ***Human Vα24 expression of lentivirus transfected Jurkat cell lines.***
A) Both cell lines, Jurkat Lucia (JL) and Jurkat 76 (JL76) were transfected with fresh produced viruses, containing the bidirectional CMV (pMJA285) or EF2 promoter + GSG-2A sequences (pMJA289) or none, maintained in normal T cell media without selection for 72 h and subsequently stained with anti-human-Vα24 for FACS analyses. % transfection expressed as the fraction of positive labelled/total cells. B) Titration curve, both cell lines, JL and J76 transduced with pMJA289, were cocultured with Mutz-3 incubated with different concentrations of α-GalCer. The supernatant was collected after 24h and IL-2 was measured in triplicate.

Furthermore, the different Jurkat Lucia and Jurkat76 cell lines were co-cultured with Mutz3 + α-GalCer and only Mutz3 (Figure 5C Jurkat Lucia, Figure 5D Jurkat 76 background). JL285 as well as J76 showed a notable activation by α-GalCer.

The expression of the human TCR by lentivirus was further monitored by luminescence and IL-2 expression in the presence of α-GalCer. In a time course experiment and as shown in Figure 5A and B the assay results from the co-culture one to four weeks post-transduction have significantly changed. The difference between the cell lines with transduced TCR (JL 285 and JL 289) were notable higher compared to JL 288 ($p<0.05$). This experiments suggest that there is only a limited time frame the transduced T-cells can be used. It has been described that after one week, the cells can undergo apoptosis and may become exhausted and lose functions with repetitive re-stimulations (Zhong et al., 2010).

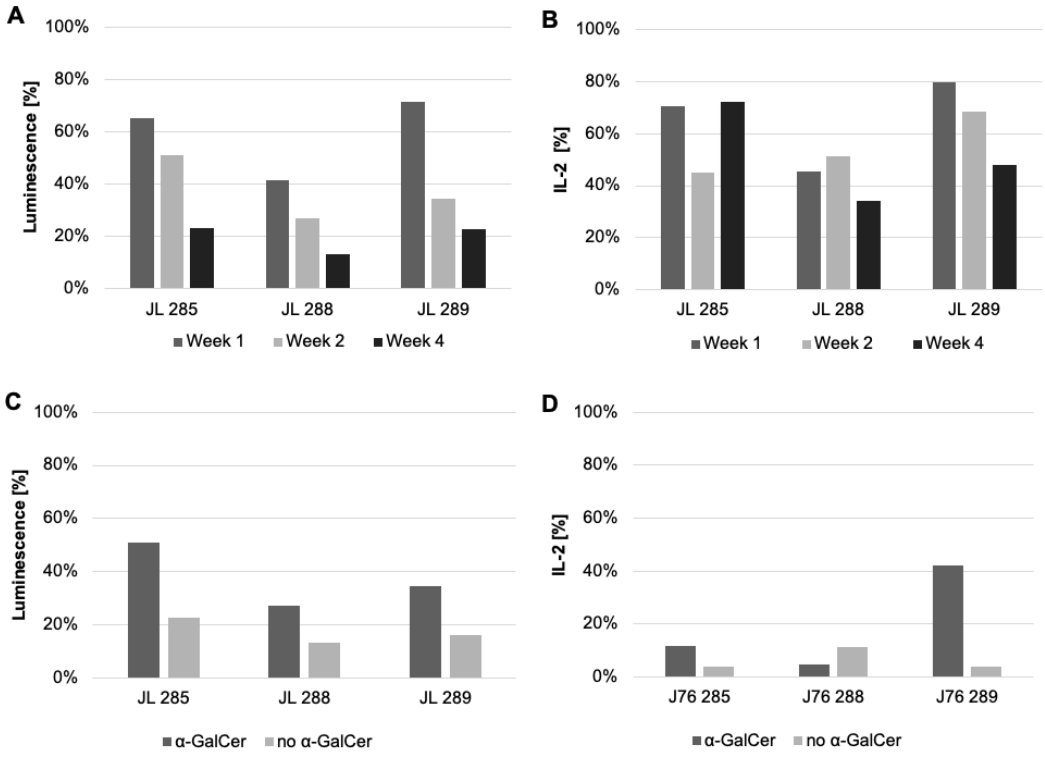


Figure 5: ***Lentiviral expression of luciferase and IL-2 after coculture***

The different Jurkat cell lines (JL:Jurkat Lucia and J76: Jurkat 76 TCR null) were transduced with pMJA285, pMJA288 (no TCR) and pMJA289. Co-culture time experiments were set Mutz3+α-GalCer for 4 weeks. A) JL time course luminescence reading. B) ibid but IL-2 measurements. C) JL 24h co-culture with Mutz3 +-α-GalCer. D) ibid using J76 cell line and IL-2 expression.

3. Conclusions

Taken together these results demonstrated that widely used murine hybridoma cell line DN32.D3 is unusable as far as expression of new TCR sequences are concerned. The results also suggested that a commercial human cell line Jurkat Lucia, containing as reporter the secreted coelenterazine luciferase regulated by human NFAT, is functional and potentially could be used for a transient expression of human TCRs, in a lipid screening program. Further, higher transfection efficiencies were obtained with the lentivirus polycistronic constructs containing the P2A sequence in a TCR $\alpha\beta/\gamma\delta$ null cell (Jurkat 76). These results suggest that indeed the mis-pairing of the endogenous α/β TCR during ER folding in the presence of the new human TCR sequences impaired the functionality of the TCR lipid receptors. These are important first steps in the

establishment of cell specific lipid responsive libraries for the study of natural lipid substances.

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