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# Towards a surrogate system to express human lipid binding TCRs

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## J Immunological methods

**Keywords:** Food allergy; NKT; TCR; Lipid binding TCR; Dendritic cells; Muc2; JawsII; *in vitro* system

## Abstract:

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

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

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## 63 Introduction

64 What makes a protein an allergen with respect to the ability to sensitise and, in  
65 particular a food allergen, is not well understood. Previously Kean et al reported that  
66 SFA8, the 2S albumin from sunflower, and not the major nut allergen Ber e 1 was able  
67 to polarize dendritic and T helper (Th) cell responses in mice with production of IL-12  
68 p40 and TNF-alpha. Transcription analysis showed increased Th1 transcription factor  
69 T-bet with respect to both proteins, but some Th2 GATA-3 with respect to Ber e 1  
70 (Kean et al., 2006). Subsequently, it was shown that Brazil nut lipids were required for  
71 sensitization with Ber e 1 (Dearman et al., 2007) and that one particular complex lipid  
72 fraction (lipid C) was able to induce specific Ber e 1 anaphylactic antibodies in naïve  
73 animals. Subsequently, in kinetic experiments we have also shown that Ber e 1 can  
74 indeed accommodate one lipid molecule (stoichiometry 1:1) with  $K_d$  of  $5.6 \pm 0.1 \mu\text{M}$   
75 (Mirotti et al., 2013; Rundqvist et al., 2012). The requirement for natural lipids from  
76 nuts as a critical component for the intrinsic allergenicity of Ber e 1 was further  
77 demonstrated when human T-cell lines derived from nut allergic patients were shown  
78 to produce IL-4 to Ber/lipid C in a CD1d dose dependent manner. Ja18 and CD1 knock  
79 out experiments further implicated Natural Killer T Cells (NKTs) in the response  
80 (Mirotti, Florsheim et al. 2013). Altogether, these results illustrate the essential role of  
81 the natural lipid fraction in nut protein sensitization and strongly suggest that NKTs  
82 play a critical role in the development of Brazil nut –allergic response (Mirotti et al.,  
83 2013).

84

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

96 via CD1 generally respond to changes in extracellular environment (Dowds et al.,  
97 2014). Within the context of allergies, NKT cells were shown to participate in Th2  
98 responses through a CD1d-dependent mechanism (Mirotti et al., 2013).

99  
100 One of the major bottlenecks in the studies of the interaction of natural lipids with the cells  
101 of the human immune system is the lack of human lipid responsive cell lines. In the present  
102 study, we attempted to engineer human TCRs into a stable murine hybridoma cell line  
103 employing well described synthetic TCR sequences and utilizing the widely used marine  
104 sponge glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) as an activator (Bai et al., 2012).  
105 Further, our work with murine and human stable DC cell lines has taken the work to  
106 commercial surrogate human T cells (Jurkats). The transient expression in several Jurkat  
107 backgrounds via plasmid or via lentivirus was also analyzed. Thus, the methodology,  
108 results and discussion presented herein will help towards a cell system able to express  
109 human responsive lipid binding TCR sequences. These are important first steps in the  
110 establishment of cell specific lipid responsive libraries for the study of natural lipid  
111 substances.

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## 113 **1. Material and methods**

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### 115 **1.1 Cell lines**

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

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## 131 1.2 Chemicals

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

144

## 145 1.3 Antibodies

146 Anti-V $\alpha$ 24PE antibodies were from Beckman Coulter. eBioscience Human IL-2 ELISA  
147 Ready-Set-Go Kit was bought from Fisher Scientific.

148

## 149 1.4 pMJA219 TRBV25/TRAV10 expression plasmid

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

162

### 163 1.5 Lentivirus expression

164 The lentivirus expression was carried out using the 3 plasmid second generation system  
165 essentially as described by Roth et al. (Roth et al., 2017). The psPAX2 (packaging) and  
166 pMD2.G (VSV-G expressing envelope) were gifts from Didier Trono (Addgene plasmid  
167 12260 and 12259 respectively). pSin-EF2-Nanog-Pur (target vector) was a gift from  
168 James Thomson (Addgene plasmid 16578). Two target vectors were assembled.  
169 pMJA285 (Genbank MH782473) contained the bidirectional CMV promoter driving  
170 TRAV10 (GenBank DQ341448.1) and TRBV25 (Genbank DQ341454.1) sequences in  
171 opposite orientation as described for the pMJA219, Xbal and Spel fragment were inserted  
172 into pSin-EF2-Nanog-Pur. pMJA289 (GenBank MH782475) was assembled by PCR  
173 overlapping primers and contained the same TRAV10/TRBV25 constructs in a dicistronic  
174 orientation driven by EF2 promoter and the  $\alpha/\beta$  TCR sequences separated by the GSG-  
175 2A self-cleavage peptide sequence GSGATNFSLLKQAGDVEDNPGP (Liu et al., 2017).  
176 For the transduction, the target cells (Jurkat Lucia or Jurkat 76) were counted. For each  
177 transduction, 105 cells were used. The cell suspension was centrifuged (1000 rpm, 5 min)  
178 in separated tubes and the supernatant was discarded. Cell pellets were resuspended in  
179 2 ml virus supernatant. Polybrene was added at a final concentration of 8  $\mu\text{g}/\text{ml}$ . The  
180 suspension was transferred into a 6 well plate. The plate was sealed with parafilm  
181 centrifuged for 90 min at 1200 rpm (spin transduction). Afterwards, the cells were carefully  
182 resuspended and incubated for another 6 hours at 37 °C. Subsequently, the cells were  
183 centrifuged and resuspended in fresh Jurkat media. The supernatant was carefully  
184 discarded and inactivated. 48 hours post transfection, the cells were split. Puromycin  
185 antibiotic was added to one well at a final concentration of 0.5  $\mu\text{g}/\text{ml}$ . One week later, the  
186 cells were tested in a co-culture. Furthermore, the cells were stained with Anti-V $\alpha$ 24PE  
187 antibodies and analysed in flow cytometry as described below.

188

### 189 1.6 Co-culture

190 DC-lipid load: human DC MUTZ-3 were cultured at 37°C, 5 % CO<sub>2</sub> in media containing  $\alpha$ -  
191 MEM with FBS (20%), condition media (5637 cell line supernatant at 20%) and human  
192 GM-CSF (5ng/ml) according to cell culture instructions (DSMZ). Mouse DC JAWSII were  
193 cultured in the same conditions but in  $\alpha$ -MEM with FBS (20%), supplemented with L-  
194 glutamine (4mM) Na-pyruvate (1mM) and murine GM-CSF (5ng/ml). For lipid loading

195 MUTZ-3 or JAWII were counted, centrifuged and resuspended in fresh DC media (RPMI  
196 1640, 10% FBS, 1% PenStrep and 5% DMSO) to a final density of  $2.5 \times 10^4$  cells/ $30 \mu\text{l}$  and  
197 added onto each well of a 96 well plate. Lipid or  $\alpha$ -GalCer was added to MUTZ or JAWSII  
198 cells to a total  $50 \mu\text{l}$ /well reaction mixture and incubated for 24h at  $37^\circ\text{C}$ , 5 %  $\text{CO}_2$ .

199 T cell transfection: Jurkat Lucia cells were cultured in Jurkat media (RPMI 1640, 10% FCS  
200 and  $100 \mu\text{g/ml}$  Zeocin) accordingly to the manufacturer's instructions (InvivoGen). The  
201 media was changed every three days, by switching between media with and without  
202 Zeocin. Jurkat 76 cells were cultured in Jurkat76 media (RPMI 1640, 10% FCS, 1% L-  
203 Glut, 1% sodium pyruvate, 1% PenStrep). For transfection, the cells were counted,  
204 centrifuged and resuspended in  $100 \mu\text{l}$ /well fresh Jurkat media to a final density of  $2 \times 10^5$   
205 cells/well and dispensed onto a 96 well plate. The transfections were performed by the  
206 procedure described in the Fugene 6 Transfection Reagent protocol (Promega,UK).  
207 Briefly, a total of  $50 \mu\text{l}$  of transfection reaction was prepared in RPMI media:  $3 \mu\text{l}$  Fugene  
208 6 reagent was added followed by  $1 \mu\text{g}$  plasmid DNA and incubated for 30 min RT. Finally,  
209 all transfection reactions were added drop-wise to each well containing Jurkat cells and  
210 incubated for 24h at  $37^\circ\text{C}$ , 5 %  $\text{CO}_2$ .

211 Co-transfection and readout: After 24h the lipid loaded MUTZ3 cells were mixed and  
212 added ( $50 \mu\text{l}$ /well) to transfected Jurkat Lucia cells onto a  $200 \mu\text{l}$  final reaction containing  
213  $2.4 \times 10^4$  DC+  $2 \times 10^5$  T cells) and incubated overnight at  $37^\circ\text{C}$ , 5 %  $\text{CO}_2$ . PMA ( $50 \text{ng/ml}$ )  
214 and ionomycin ( $3 \mu\text{g/ml}$ ) were used as positive controls to stimulate T cells. After 24h  
215 incubation, the supernatant was harvested, and the secreted luciferase activity measured  
216 by QUANTI-Luc assay containing coelenterazine substrate. For this,  $20 \mu\text{l}$  of each sample  
217 was transferred onto Optiplate 96 plate,  $50 \mu\text{l}$ /well QUANTI-Luc added and immediately  
218 measured onto a luminometer (Turner Biosystems) and analysed in MS-Excel. The  
219 remaining supernatant was stored at  $-20^\circ\text{C}$  or used in an IL-2 Sandwich ELISA  
220 determination (384 well plate) using HRP and TMB as substrate following the Ready-Set-  
221 Go! Kit according to the manufacturer's instructions (Fisher Scientific). The results were  
222 read at 450 nm using Tecan Infinite M200 PRO plate reader, data collected by Magellan  
223 software and transferred to a MS-Excel spreadsheet for analysis.

224

## 225 1.7 Flow cytometric analysis

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

228 300g. Supernatants were discarded, and cell pellet was re-suspended. 5 $\mu$ l antibodies (anti  
229 human V $\alpha$ 24 antibody) were added and incubated at 4°C for 30 min in the dark.  
230 Afterwards, 2 ml of PBA was added to each tube. Supernatants were discarded, pellet re-  
231 suspended and cells were fixed in 0.5 ml PBA. Analysis was carried out on the Flow  
232 Beckman Coulter flow cytometer FC500 (Beckman Coulter life Sciences, USA), at the  
233 Flow Cytometry Facility (University of Nottingham). After quantification by FC500 the data  
234 was analysed using Kaluza software (Beckman Coulter).

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## 239 **2. Results and discussion**

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### 242 2.1 Lipid responsive TCR, murine system

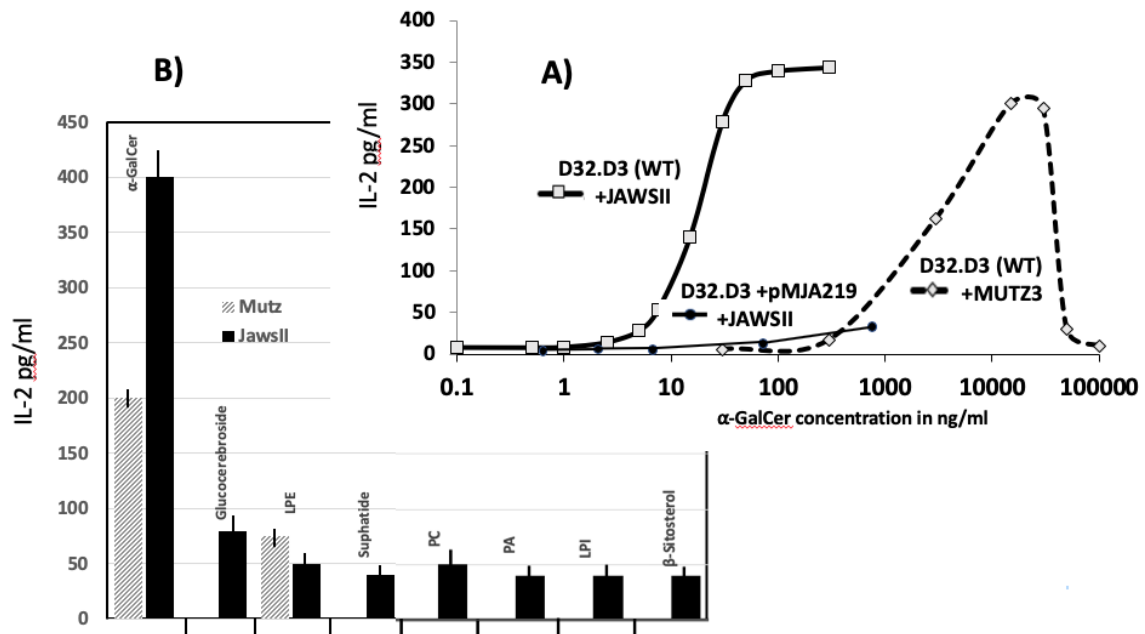
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244 The murine hybridoma cell line DN32.D3, originally described by A. Bendelac (Lantz and  
245 Bendelac, 1994), has been widely used by many groups working with lipid binding and  
246 NKT cell activation. The TCR of DN32.D3 cell line consists of V $\alpha$ 14 J $\alpha$ 18 paired with V $\beta$ 8,  
247 V $\beta$ 7 or V $\beta$ 2 and the cell line expresses the NK1.1 marker, an identifier of NK cells. The  
248 characteristics of DN32.D3 cell line have been described in great detail elsewhere  
249 (Jordan-Williams et al., 2013; Kim et al., 2010, 2006). This double negative hybridoma  
250 murine cell line when presented in co-culture to the murine DC cell line JawsII has shown  
251 a sigmoidal IL-2 dose response curve with increasing  $\alpha$ -GalCer concentration (Figure 1A).  
252 This response is blocked by murine anti-CD1d antibodies, hence CD1d specific, and is  
253 not further amplified by murine anti-CD3/CD28 beads. Furthermore, the response  
254 seemed to be IL-2 specific as only the IL-2 cytokine was detected, however, a Luminex  
255 panel containing only 6 cytokines (IL-2, 4, 5, 10, 12 and IFN- $\gamma$ ) was used (results not  
256 shown).

257 As expected, the dose response curve of DN32.D3 against  $\alpha$ -GalCer in co-culture is  
258 species-dependent. DN32.D3 showed a higher sensitivity to lower levels of  $\alpha$ -GalCer  
259 when the glycolipid is presented by the murine JAWSII than when presented by the human  
260 MUTZ3 DC cell lines (Figure 1B). Regarding lipid specificity, DN32.D3 has shown to be  
261 quantitatively specific to  $\alpha$ -GalCer in the murine JAWSII system (Ghumra and Alcocer,  
262 2017) with a high background response for the other lipids tested (Figure 1A). The human



263 DC system MUTZ3, although less sensitive, responded in addition to  $\alpha$ -GalCer to LPE  
 264 and SDS (Figure 1B).  
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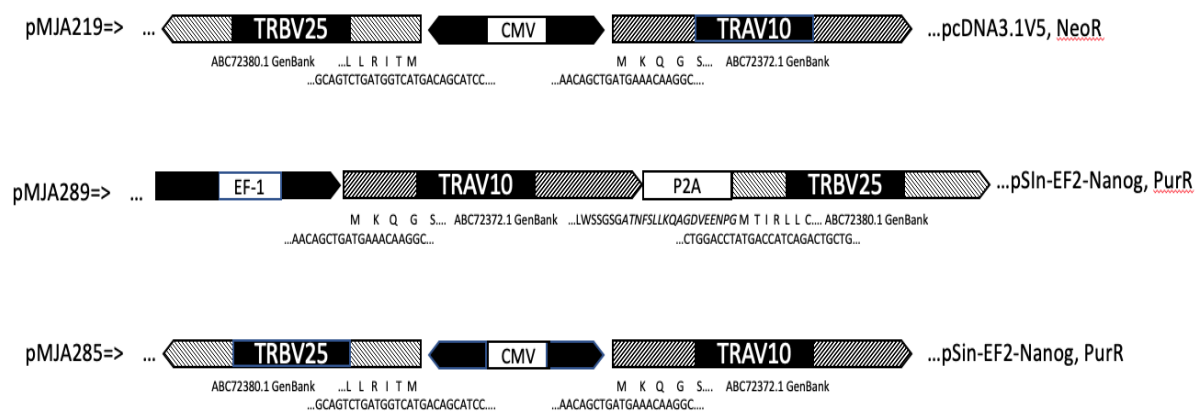


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 267 **Figure 1:  $\alpha$ -GalCer dose response of DN32.D3 co-cultured with JAWSII or MUTZ3 cell lines.** A) IL-2- $\alpha$ -  
 268 GalCer dose response of DN32D3 wild type and transfected DN32.D3 with the plasmid pMJA219 with JAWSII  
 269 or MUTZ3 as indicated after 72h 37°C. B) IL-2-lipid specificity response for DN23.D3 and MUTZ3 or JAWSII,  
 270 lipids at 1ug/ml for JAWSII and 10ug/ml for MUTZ3. LPE: lyso-Phosphatidyl ethanolamine, PC: phosphatidyl  
 271 choline, PA: Phosphatidic acid, LPI: lyso-phosphatidyl inositol. All co-cultures were performed at density of  
 272  $5 \times 10^6$  cells/well of DN32.D3 and  $5 \times 10^4$  cells/well of APC.  
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274  
 275 In an attempt to engineer new functions and humanise DN32.D3, a plasmid (pMJA219)  
 276 containing the  $\alpha$ -GalCer responsive human TRAV10 and TRBV25 sequences was  
 277 constructed (Fig 2). The  $\alpha/\beta$  TCR sequences used in pMJA219 design have been  
 278 previously described as  $\alpha$ -GalCer specific using lipid loaded tetramers (Brigl et al., 2006).  
 279 In order to achieve TCR  $\alpha/\beta$  equimolar expression a mammalian pMJA219 plasmid was  
 280 designed that contained the bidirectional CMV promoter driving the TCR sequences. After  
 281 transfection and selection for 3 weeks on Gentamicin, FACS analysis using TRAV10  
 282 specific human antibodies (V24 $\alpha$  sequence) confirmed that 60-80% of pMJA219 DN32.D3  
 283 transfected cells have displayed the specific human TRAV10 and none on the controls.  
 284 These results confirmed that the bidirectional promoter is functional and that the murine  
 285 cell line DN32.D3 can express a human  $\alpha$  chain. However, when the pMJA219 transfected  
 286 DN32.D3 cells were co-cultured with the murine DC JawsII they did not show any

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

290  
 291 Whether these results were a product of competition with the murine CD3 as suggested  
 292 by some authors (Ahmadi et al., 2011) or by mis-pairing with the endogenous murine  $\alpha/\beta$   
 293 TCR during ER folding (Knies et al., 2016; Sommermeyer et al., 2006) remains to be  
 294 clearly demonstrated. What is clear from this exercise is that the over expression of the  
 295 human  $\alpha/\beta$  chains clearly disrupted the function of the endogenous murine chains (Fig  
 296 1A).



297  
 298 **Figure 2: Diagrammatic TCR clones designed and constructed for this work.** pMJA219  
 299 is a mammalian NeoR plasmid (GenBank MH782476) driven by a bi-directional CMV promoter  
 300 (pBI-CMV1, Clontech). pMJA285 (GenBank MH782473) is a similar construct but cloned into  
 301 the lentivirus pSin-EF2-Nanog, PurR background. pMJA289 is a polycistronic construct into  
 302 which the P2A cleavage sequence has been engineered. The AA proteolytic cleavage  
 303 sequence of the P2A is indicated in italic. pMJA288 is pSin-EF2-Nanog lentivirus vector used  
 304 in the cloning but without any insert.

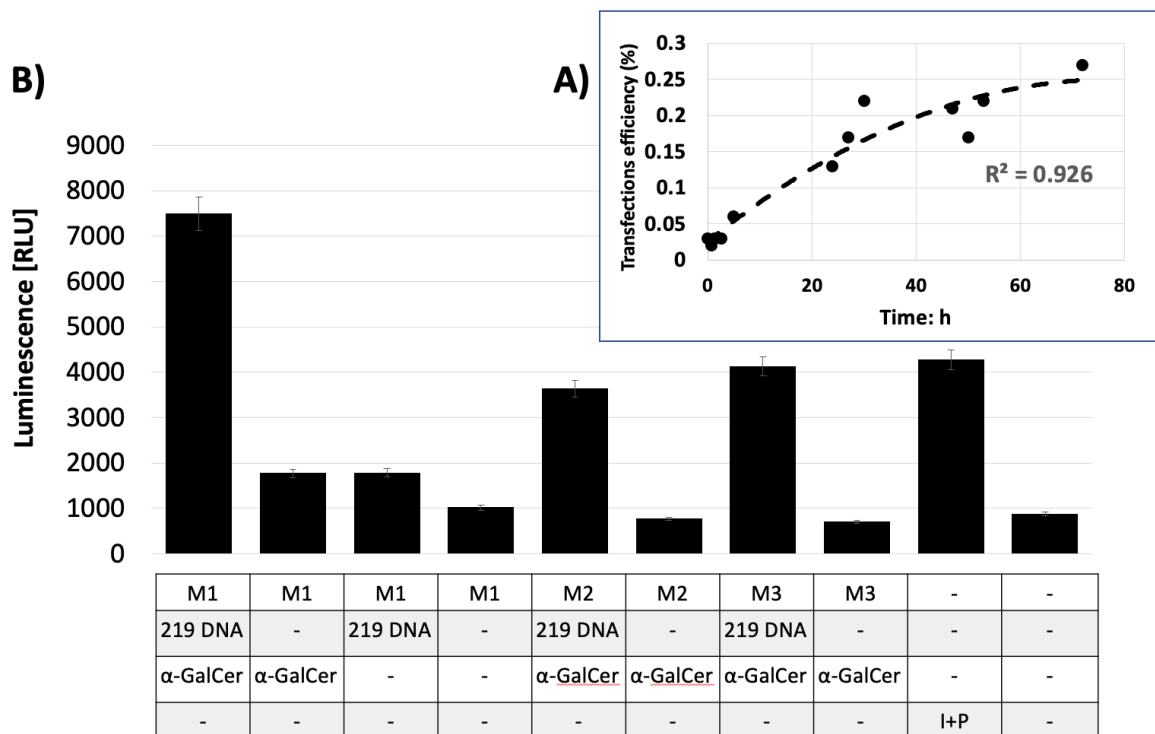
## 305 2.2 Jurkat Lucia cells

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

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

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

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*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 V24 $\alpha$  by FACS. B) Luminescence readings of triplicate wells after 24h of co-incubation of MUTZ3 and Jurkat Lucia cells at density of  $2 \times 10^5$  each/well at 37°C, 5 % CO<sub>2</sub>. 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  $\alpha$ -MEM 60%, 20%FBS, 100ng/ml hGM-CSF, 10ng/ml IL-4, 75ng/ml TNF- $\alpha$  (Ning et al., 2011). M3=lipid+24h in alpha-MEM 60%, 20%FBS, 50ng/ml hGM-CSF, 20ng/ml IL-4, 12ng/ml TNF- $\alpha$  (Masterson et al., 2002). I+P=Ionomycin+PMA.*

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

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### 2.3 Lentivirus system

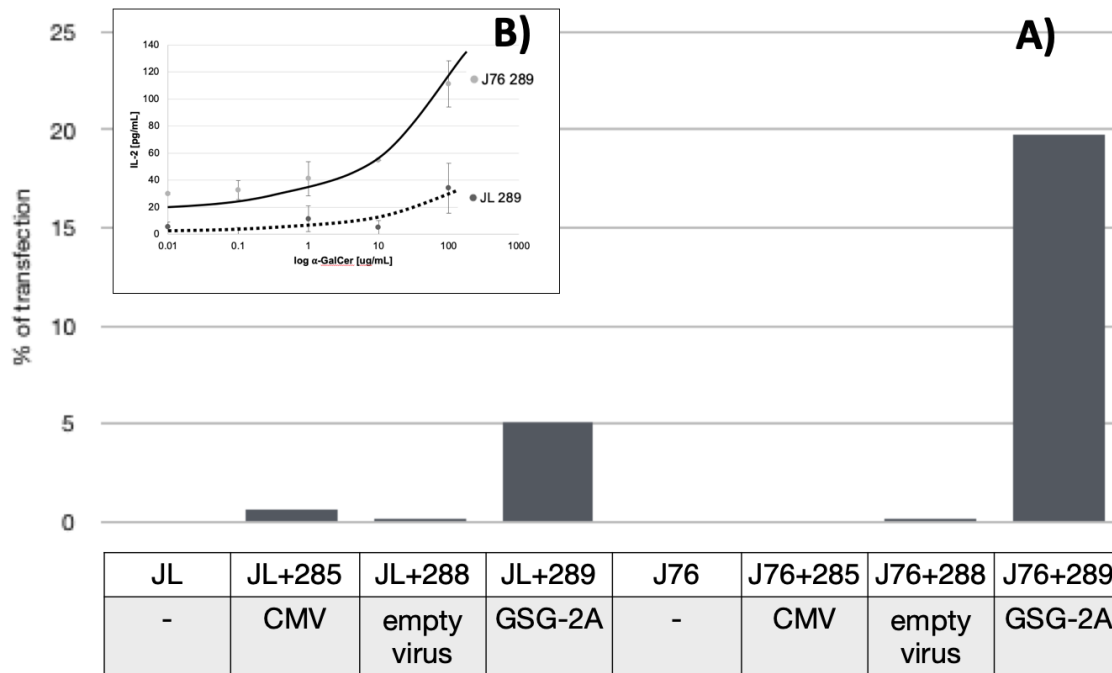
358 One alternative for plasmid driven TCR expression in T lymphocytes is the use of a  
359 lentivirus expression system as previously reported (Zhou and Buchholz, 2013). In this  
360 plasmid system the packaging and envelope genes are mixed with the target or transfer  
361 plasmid (3:1:3) and the virus packed in receptor cells. For this, the target plasmid  
362 pMJA285 has been designed and constructed that contained the  $\alpha\beta$  TRAV10/TRBV25  
363 sequences and similarly to pMJA219, was driven by the CMV bidirectional promoter  
364 (Figure 2). In another strategy the target plasmid pMJA289 (Figure 2) was produced  
365 containing the  $\alpha\beta$  genes in a discistronic configuration separated by the GSG-2A self-  
366 cleavage sequence, as described by Liu et al. (2017). When these viruses containing  $\alpha\beta$   
367 TCR sequences were transfected into the Jurkat Lucia background, the dicistronic  
368 construct showed a higher transfection rate than the CMV construct after 72h, as  
369 monitored by FACS using anti human V $\alpha$ 24 Ab (Figure 4A). The human TCR expression  
370 achieved with the protease cleavage construct containing the P2A sequence (pMJA289)

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

374 Interestingly, much higher transfection efficiency with the same virus constructs was  
375 obtained with the cell line Jurkat76 when compared with Jurkat Lucia (Figure 4),  
376 suggesting a more efficient assembling of the TCR/CD3 complex in this cell line. Jurkat  
377 76 is a human TCR  $\alpha\beta/\gamma\delta$  null cell kindly donated by M. Heemskerk (Heemskerk et al.,  
378 2003) that has been reported as good and stable recipient for new TCR sequences (Guo  
379 et al., 2016). Thus, the results presented here corroborated the findings from both groups  
380 and suggests that indeed the mispairing of the endogenous  $\alpha/\beta$  TCR during ER folding in  
381 the presence of the new human TCR sequences impaired the functionality of the lipid  
382 receptors.

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

391



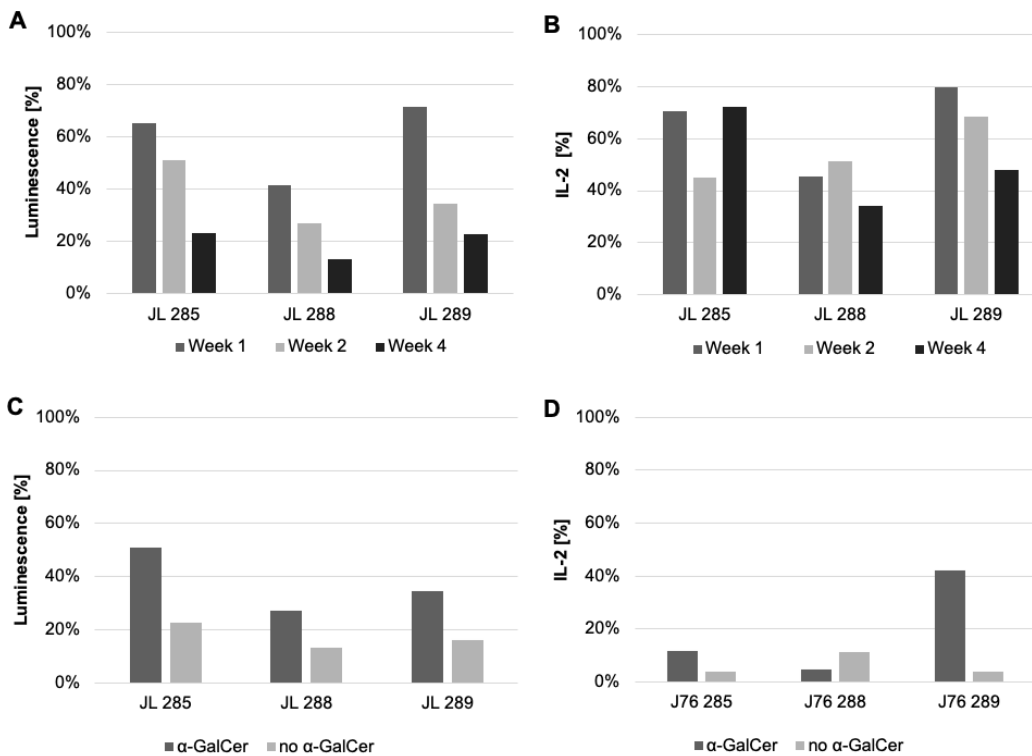
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396 **Figure 4: Human V $\alpha$ 24 expression of lentivirus transfected Jurkat cell lines.**

397 A) Both cell lines, Jurkat Lucia (JL) and Jurkat 76 (JL76) were transfected with fresh produced viruses,  
398 containing the bidirectional CMV (pMJA285) or EF2 promoter + GSG-2A sequences (pMJA289) or  
399 none, maintained in normal T cell media without selection for 72 h and subsequently stained with anti-  
400 human-V $\alpha$ 24 for FACS analyses. % transfection expressed as the fraction of positive labelled/total cells.  
401 B) Titration curve, both cell lines, JL and J76 transduced with pMJA289, were cocultured with Mutz-3  
402 incubated with different concentrations of  $\alpha$ -GalCer. The supernatant was collected after 24h and IL-2  
403 was measured in triplicate.  
404  
405

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

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



419  
 420 **Figure 5: Lentiviral expression of luciferase and IL-2 after coculture**  
 421 The different Jurkat cell lines (JL: Jurkat Lucia and J76: Jurkat 76 TCR null) were transduced with pMJA285,  
 422 pMJA288 (no TCR) and pMJA289. Co-culture time experiments were set Mutz3+ $\alpha$ -GalCer for 4 weeks. A)  
 423 JL time course luminescence reading. B) *ibid* but IL-2 measurements. C) JL 24h co-culture with Mutz3 + $\alpha$ -  
 424 GalCer. D) *ibid* using J76 cell line and IL-2 expression.

425

### 426 3. Conclusions

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

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

439

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444

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