The detailed distribution of T cell subpopulations in immune-stable renal allograft recipients: a single center study

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Background: Most renal allograft recipients reach a stable immune state (neither rejection nor infection) after transplantation. However, the detailed distribution of overall T lymphocyte subsets in the peripheral blood of these immune-stable renal transplant recipients remains unclear. We aim to identify differences between this stable immune state and a healthy immune state. **Methods:** In total, 103 recipients underwent renal transplantation from 2012 to 2016 and received regular follow-up in our clinic. A total of 88 of these 103 recipients were enrolled in our study according to the inclusion and exclusion criteria. Forty-seven patients were 1 year post-transplantation, and 41 were 5 years post transplantation. In addition, 41 healthy volunteers were recruited from our physical examination clinic. Detailed T cell subpopulations from the peripheral blood were assessed via flow cytometry. The parental frequency of each subset was calculated and compared among the diverse groups. **Results**: The demographics and baseline characteristics of every group were analyzed. The frequency of total T cells (CD3+) was decreased in the renal allograft recipients. No difference in the variation of the CD4+, CD8+, and activated (HLA-DR+) T cell subsets was noted among the diverse groups. Regarding T cell receptor (TCR) markers, significant reductions were found in the proportion of γδ T cells and their Vδ2 subset in the renal allograft recipients. The proportions of both CD4+ and CD8+ programmed cell death protein (PD) 1+ T cell subsets were increased in the renal allograft recipients. The CD27+CD28+ T cell proportions in both the CD4+ and CD8+ populations were significantly decreased in the allograft recipients, but the opposite results were found for both CD4+ and CD8+ CD27-CD28- T cells. An increased percentage of CD4+ effector memory (EM) T cells and a declined fraction of CD8+ central memory (CM) T cells were found in the renal allograft recipients. **Conclusion**: Limited differences in general T cell subsets (CD4+, CD8+ and HLA-DR+) were noted. However, obvious differences between renal allograft recipients and healthy volunteers were identified with TCR, PD1, costimulatory molecules and memory T cell markers.

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2   **renal allograft recipients: a single center study**

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29 **ABSTRACT**

30 **Background:** Most renal allograft recipients reach a stable immune state (neither rejection nor

31 infection) after transplantation. However, the detailed distribution of overall T lymphocyte

32 subsets in the peripheral blood of these immune-stable renal transplant recipients remains unclear.

33 We aim to identify differences between this stable immune state and a healthy immune state.

34 **Methods:** In total, 103 recipients underwent renal transplantation from 2012 to 2016 and

35 received regular follow-up in our clinic. A total of 88 of these 103 recipients were enrolled in our

36 study according to the inclusion and exclusion criteria. Forty-seven patients were 1 year post-

37 transplantation, and 41 were 5 years post transplantation. In addition, 41 healthy volunteers were

38 recruited from our physical examination clinic. Detailed T cell subpopulations from the

39 peripheral blood were assessed via flow cytometry. The parental frequency of each subset was

40 calculated and compared among the diverse groups.

41 **Results**: The demographics and baseline characteristics of every group were analyzed. The

42 frequency of total T cells (CD3+) was decreased in the renal allograft recipients. No difference in

43 the variation of the CD4+, CD8+, and activated (HLA-DR+) T cell subsets was noted among the

44 diverse groups. Regarding T cell receptor (TCR) markers, significant reductions were found in

45 the proportion of γδ T cells and their Vδ2 subset in the renal allograft recipients. The proportions

46 of both CD4+ and CD8+ programmed cell death protein (PD) 1+ T cell subsets were increased in

47 the renal allograft recipients. The CD27+CD28+ T cell proportions in both the CD4+ and CD8+

48 populations were significantly decreased in the allograft recipients, but the opposite results were

49 found for both CD4+ and CD8+ CD27-CD28- T cells. An increased percentage of CD4+ effector

50 memory (EM) T cells and a declined fraction of CD8+ central memory (CM) T cells were found

51 in the renal allograft recipients.

52 **Conclusion**: Limited differences in general T cell subsets (CD4+, CD8+ and HLA-DR+) were

53 noted. However, obvious differences between renal allograft recipients and healthy volunteers

54 were identified with TCR, PD1, costimulatory molecules and memory T cell markers.

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59 **INTRODUCTION**

60 Renal transplantation is still the most applicable management strategy for end-stage renal disease

61 (ESRD) (*Darres et al., 2018*). Every patient needs to take immunosuppressants (ISs) to prevent

62 rejection after organ transplantation, but taking an overdose of ISs will cause infection and drug

63 toxicity (*Mota et al., 2013*). After a period of continuous monitoring and adjustment of the IS

64 concentration and immunocompetency, renal allograft recipients can achieve a stable immune

65 state (neither infection nor rejection). However, this stable immune state is complex and is

66 influenced by multiple factors, such as ISs, allograft immune activity and psychological changes.

67 Therefore, this immune-stable state should be different from healthy immunity.

68 Renal allograft recipients mainly take calcineurin inhibitors (CNIs) to prevent cellular rejection

69 responses after transplantation (*Yu et al., 2018*). The most commonly applied CNI in our

70 department is tacrolimus (also known as FK506). The immunosuppressive activities of

71 tacrolimus and other commonly used ISs (e.g., mycophenolate mofetil (MMF) and steroids)

72 mainly impact T lymphocytes (*Hartono, Muthukumar & Suthanthiran, 2013*). Therefore, the

73 constitution and proportion of T cells and their subpopulations should receive considerable

74 attention. The chief subpopulations of T cells are indicated by the cell surface markers CD4 and

75 CD8 together with CC chemokine receptor 7 (CCR7) and CD45RA (*Busch et al., 2016*). The

76 activated subpopulations of each of these cell types can be defined by the supplement of

77 activation markers, such as CD38 and HLA-DR (*Ferreira, Kumar & Humar, 2018; Tanko et al.,*

78 *2018*). The naive T cell, effector memory (EM), central memory (CM), and effector T cell

79 subsets were first defined based on CCR7 and CD45RA expression (*Sallusto et al., 1999*). The

80 specific mechanism for the above ISs is inhibition of the production of interleukin (IL)-2, which

81 allows naïve T cells to differentiate into effector T cells in lymphatic tissues (*Hartono,*

82 *Muthukumar & Suthanthiran, 2013*). Compared with the activation process in naïve T cells,

83 memory T cells are activated by antigen-presenting cells in diverse tissues, including organ

84 allografts and are less susceptible to suppression by immunosuppressive agents and tolerogenic

85 cells (*Yang et al., 2007*), whereas allo-reactive CD8+ memory T cells are known to be more

86 resistant than CD4+ T cells (*Perez-Gutierrez et al., 2018*). Both CM and EM T cells show

87 potential for generation following acute allograft rejection (*Danger, Sawitzki & Brouard, 2016;*

88 *Siu et al., 2018*). Additionally, T cells need 2 signals for activation: the T cell receptor (TCR)

89 and costimulatory molecules (*Agarwal & Newell, 2008*). There are 2 major categories of

90 costimulatory molecules: the immunoglobulin superfamily (i.e., CD28) and the tumor necrosis

91 factor receptor superfamily (i.e., CD27) (*Croft, 2003*). More recently, T cell immunosenescence

92 has been shown to related to telomere-dependent replication senescence, and its characteristic

93 phenotype and functional spectrum are associated with CD57 expression(*Cura Daball et al.,*

94 *2018*), which is also associated with altered function (*Larbi & Fulop, 2014*). These CD57+ T

95 cells were initially defined as being unable to proliferate under antigen stimulation, but recent

96 studies showed that they could enter the active cell cycle and proliferate under certain

97 stimulation conditions and maintain cytokine production (*Strioga, Pasukoniene & Characiejus,*

98 *2011*). Several studies clearly demonstrated that CD8+CD57+ T cells showed

99 immunosuppressive activity and were more active in immunosuppressant-treated and human

100 immunodeficiency virus (HIV)-infected patients (*Frassanito et al., 1998; Sadat-Sowti et al.,*

101 *1994*). The programmed cell death protein 1 (PD1) plays a role in chronic infection and organ

102 transplant tolerance, and PD1 upregulation is associated with T cell exhaustion phenotypes in

103 multiple animal models (*Wang, Han & Hancock, 2007*).

104 Continuous monitoring of immune cells is important for disease treatment and prognostic

105 prediction (*Goldschmidt et al., 2018*). The peripheral blood is very important for assessing the

106 immune state, because it is convenient to be collected and contains abundant significant

107 information concerning the arrival of immune cells in the cognate tissue through the circulation

108 (*Ruhle et al., 2016*). Multicolor flow cytometry is considered the preferred scheme for analysis

109 of blood samples, because it provides highly specific single cell levels with various indexes and

110 high output characteristics (*Streitz et al., 2013*).

111 In our study, we obtained peripheral blood specimens from recipients 1 and 5 years post-kidney

112 transplantation and analyzed their detailed T cell subset immunophenotyping using multicolor

113 flow cytometry. We compared these profiles with those of healthy volunteers to assess the

114 constitution and frequency of T cell subpopulations in immune-stable recipients.

115

116 **METHODS AND MATERIALS**

117 **Study population and blood specimen collection**

118 In total, 103 recipients aged 18-65 years underwent kidney transplantation from 01.01.2012 to

119   12.31.2016 and received regular follow-up in the clinic of the 3rd Xiangya Hospital, Central

120   South University. A total of 88 of these 103 recipients were eligible to be collected as cases for

121   further investigation according to the key inclusion and exclusion criteria (Table 1). The

122   recipients were allocated into 2 groups dependent on their postoperative period. In total, 47

123   patients were 1 year post-transplantation (1-year group), and 41 patients were 5 years post-

124   transplantation (5-year group). All of the patients had experience with dialysis prior to

125   transplantation. Additionally, 41 healthy volunteers were recruited as a control group (healthy

126   group) from our physical examination clinic. All participants provided written informed consent.

127   The study protocol was reviewed and approved by the institutional review board (Ethics

128   Committee) of the 3rd Xiangya Hospital, Central South University (No. 2018-S347).

129   One mL of peripheral blood was obtained in a vacuum tube (BD, Heidelberg, Germany)

130   containing ethylenediaminetetraacetic acid (EDTA) for anticoagulation. All samples were tested

131   immediately or stored at room temperature for no more than one hour after collection.

132

133   **Leukocyte staining**

134   Flow cytometric fluorescent anti-human monoclonal cell surface antibody (dry powder) tubes

135   (DuraClone IM) were purchased from Beckman Coulter (Bangalore, India). The details of every

136   fluorochrome-conjugated antibody, the schemes of every fluorochrome channel and the

137   compensation controls (each of a single color) are presented in Table 2. Briefly, 100 μL of

138   anticoagulant blood was stained with fluorescent antibodies for 15 minutes in the dark (room

139   temperature). The erythrocytes were removed by adding 2 mL of lyse-fix solution consisting of

140   Versa Lyse^{TM} and IOTest^{VR} Fixative Solution (MBL Life Science, Japan) and incubated for 15

141   minutes in the dark (room temperature). Then, the cells were rinsed twice and resuspended in

142   staining buffer (phosphate-buffered saline containing 2% fetal bovine serum) prior to acquisition.

143   All samples were analyzed with a 13-Color CytoFlex Flow Cytometer (Beckman Coulter) after

144   daily calibration with Flow-Set Pro Beads (Beckman Coulter).

145

146   **Data analysis**

147   The collected flow cytometric information was investigated using the Kaluza Software version

148   1.2 (Beckman Coulter) by a single operator according to the ONE-Study protocol (*Streitz et al.,*

149 *2013*). For setting up compensation using the AutoSetup Scheduler, refer to the Application Note

150 "Compensation Setup for High Content DuraClone reagents", which is downloadable from the

151 Beckman Coulter website (www.duraclone.com). The adhesive doublets were removed by two

152 forward scatter parameters (width versus height). We used CD45 and side scatter to gate

153 leukocytes. The parameters used to calculate the size and frequency of the subsets were exported

154 from the Kaluza software into Excel (Microsoft, Redmond, WA, USA).

155

156 **Statistical Analysis**

157 The mean±standard deviation (SD) was used to describe the analyzed data. At baseline, the

158 proportion gender ratio was compared with the Chi-square test of independence, and the mean

159 age and serum creatinine levels were compared by one-way ANOVA. The FK506 concentration

160 was compared by the Mann–Whitney U test. Differences in T lymphocyte subset percentages

161 among the groups were compared using the Mann–Whitney U test, because not all of the

162 parameters were distributed normally. GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla,

163 CA, USA) was used to perform the statistical analyses. Values of $p<0.05$ were considered

164 statistically significant. * indicates $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

165

166 **RESULTS**

167 **Demographic and Baseline Characteristics**

168 The demographic and baseline characteristics of each group are presented in Table 3. The

169 patients in the 5-year group exhibited an increased mean age (44.53±10.02) compared with that

170 of the 1-year group. More male patients were noted in the 1-year and 5-year groups. All

171 participants in our study had peripheral WBC counts (95% confidence interval [CI]: 7.035~7.625)

172 and lymphocytes counts (95% CI: 1.724~1.950) within the normal ranges. All participants in our

173 study exhibited normal serum creatine ranges (95% CI: 93.20~103.1). All patients post-kidney

174 transplantation took FK506+MMF+ prednisolone (Pred). The cytomegalovirus (CMV) status

175 was not assessed in this study, because almost all of the enrolled allograft recipients were

176 serologically CMV-positive, and only four of the patients were negative.

177 **T cell subset gating strategies**

178   Based on the details of each T cell subpopulation shown in Fig. 1, we used the forward scatter

179   area and CD45 to gate peripheral lymphocytes. CD3+ cells were defined as T cells and divided

180   into 5 populations using the CD4, CD8, HLA-DR and TCR cell markers as follows: 1) CD4+ T

181   cells, 2) CD8+ T cells, 3) HLA-DR+ T cells, 4) αβ T cells, and 5) γδ T cells. The γδ T cells were

182   categorized into Vδ1 and Vδ2 subsets. Additionally, the CD8+ and CD4+ T cells were divided

183   into 8 subgroups as follows: CD57+ subset, PD1+ subset, costimulatory molecule

184   (CD28+CD27+ and CD28-CD27-) subsets, and central memory (CD45RA-CCR7+), effector

185   memory (CD45RA-CCR7-), naïve (CD45RA+CCR7+) and effector (CD45RA+CCR7-) cell

186   subsets.

187

188   **Distribution of the general T cell subsets**

189   Generally, CD3+ T cells and the two main T cell subsets (CD8+ and CD4+) are tested. We also

190   used HLA-DR to assess the T cell activation status. We observed a significant reduction in the

191   proportion of CD3+ T cells in the 1-year and 5-year kidney transplant patients compared with

192   that of the healthy volunteers. However, no significant difference was noted between the 1-year

193   and 5-year patients. No significant differences in the CD4+, CD8+ and HLA-DR+ T cells were

194   noted among the diverse groups (Fig. 2). Altogether, because only limited differences in general

195   T cell subsets were found between the healthy volunteers and allograft recipients, we needed to

196   identify more detailed T cell subpopulations. All of the means±SDs and P values are displayed in

197   Table 4.

198

199   **Distribution of the TCR T cell subsets**

200   To obtain more details for more specific T cell subsets, we applied TCR markers to define some

201   subsets. We identified a significant decline in the proportion of γδ T cells but an increased

202   proportion of αβ T cells in the renal allograft recipients compared with those of the healthy

203   volunteers. Similar results were observed for the Vδ2 and Vδ1 subsets of the γδ T cells. No

204   significant differences were found in any of the TCR subsets described above between the 1-year

205   and 5-year renal allograft recipients (Fig. 3). Taken together, obvious differences were noted

206   among the TCR T cell subsets between the healthy individuals and kidney transplant recipients.

207   All of the means±SDs and P values are displayed in Table 4.

208

Distribution of the CD57+ and PD1+ T cell subsets

209

210   CD57 and PD1 are typical cell surface markers for T cell immune senescence and regulation and

211   thus are also considered good cell surface markers for immunosuppression and tolerance,

212   respectively. In the CD4+ subsets, the percentage of CD57+ T cells was highest in the 1-year

213   renal allograft recipients compared with those of the healthy individuals and 5-year recipients.

214   No significant difference was found between the healthy volunteers and 5-year renal allograft

215   patients. Additionally, no significant differences were noted in the CD8+ CD57+ T cells among

216   the groups. The percentages of PD1+T cells in both the CD4+ and CD8+ populations were

217   significantly increased in the renal allograft recipients compared with those of the healthy

218   volunteers. Nevertheless, no significant difference was found between the 1-year and 5-year

219   renal allograft recipients (Fig. 4). All of the means±SDs and P values are displayed in Table 4.

220

Distribution of the costimulatory molecule T cell subsets

221

222   In the costimulatory molecule (CD27 and CD28) subsets, only the CD27 and CD28 double-

223   positive and double-negative subsets exhibited significant differences. The percentages of

224   CD27+CD28+ T cells in both the CD4+ and CD8+ populations were obviously decreased in the

225   renal allograft recipients compared with those of the healthy volunteers. The CD4+

226   CD27+CD28+ T cells were reduced in the 1-year compared with the 5-year recipients. In

227   contrast, the percentages of CD27 and CD28 double-negative T cells in both the CD4+ and

228   CD8+ populations were significantly increased in the renal allograft recipients compared with

229   those of the healthy volunteers. CD27 and CD28 double-negative CD4+ T cells were increased

230   in the 1-year over the 5-year recipients. No obvious differences in both the CD27 and CD28

231   double-negative and -positive T cells in the CD8+ subsets were noted between the 1-year and 5-

232   year renal allograft recipients (Fig. 5). All of the means±SDs and P values are displayed in Table

233   4.

234

Distribution of the memory T cell subsets

235

236   We observed a significantly increased percentage of CD4+ EM T cells but a decreased

237   percentage of CD8+ CM T cells in the renal allograft recipients compared with those of the

238 healthy volunteers. However, the differences in the above memory T cell subsets were not
239 significant between the renal allograft recipients and healthy volunteers (Fig. 6). All of the
240 means±SDs and P values are displayed in Table 4.

241

242 **DISCUSSION**
243 In our study, we used multicolor flow cytometry to analyze the detailed T cell subpopulation
244 immunophenotyping of peripheral blood specimens from 88 renal transplant patients 1 year and
245 5 years after transplantation and compared these results with those of healthy volunteers. The
246 distribution of T cell subpopulations in immune-stable allograft recipients was identified.
247 The fraction of total T (CD3+) cells declined in the renal allograft recipients. The percentage of
248 CD8+ T cells exhibited an increasing trend in the renal allograft recipients compared with that of
249 the healthy volunteers; however, the difference was not statistically significant. HLA-DR
250 expression, which might be activation-dependent, was also assessed (*Arneth, 2018*). However, in
251 our study, no significant differences in HLA-DR+ T cell subsets were noted. This finding may be
252 attributed to triggering of a few T cells in the immune-stable patients and healthy volunteers.
253 γδ T cells are a small subsection (3-5%) of T cells in human peripheral blood. According to their
254 TCR variable (V) gene fragment, there are two main subgroups (Vδ1 and Vδ2). Vδ2 T cells are
255 the major γδ T cell population in circulation, accounting for 50-95% of the γδ T cells in
256 peripheral blood mononuclear cells (PBMCs) (*Peters, Kabelitz & Wesch, 2018*). Although some
257 evidence indicates that γδ T cells can apply immunosuppressive features, the majority of γδ T
258 cell activities are pro-inflammatory immune responses (*Tyler et al., 2015; Xu et al., 2018*).
259 Furthermore, Vδ2 T cells can give rise to Th1-, Th2- (*Wesch, Glatzel & Kabelitz, 2001*), Th9-
260 (*Peters et al., 2016*), Th17- (*Caccamo et al., 2011*), Tfh- (*Bansal et al., 2012*) and antigen-
261 presenting cell (APC)-like phenotypes (*Brandes, Willimann & Moser, 2005*). These findings
262 potentially explain why significantly decreased proportions of γδ and Vδ2 T cells were noted in
263 the immune-stable renal allograft recipients. Additionally, we presented the percentages of Vδ2-
264 and Vδ1-positive cells as a percentage of the CD3+ cells and not simply as a percentage of the γδ
265 T cells. We found that the percentage of CD3+ Vδ2 cells in the controls was significantly higher
266 than that in the allograft recipients. However, no significant difference in CD3+ Vδ1 cells was
267 noted among the three groups (Supplementary Fig. 1).

268 CD57 was first reported as a marker of natural killer (NK) cells (*Kared et al., 2016*). CD57 is
269 present in CD8 and CD4 T cells at the late stages of differentiation and usually is applied for
270 identification of terminally differentiated "senescent" cells with a lower proliferative ability and
271 altered characteristics (*Brenchley et al., 2003*). In this paper, the proportion of CD4+CD57+ T
272 cells was increased in the renal allograft recipients, indicating that more "senescent and
273 exhausted" T cells were present in the immune-stable allograft recipients. Moreover, patients in
274 the 1-year group exhibited an increased proportion of CD4+CD57+ T cells compared with that
275 of the 5-year group, demonstrating more "senescent" T cells in the short-term patients post-
276 kidney transplantation. CD279 (PD1) makes a significant contribution to the balance of T cell
277 immunity and immune tolerance and binds its ligand (PDL1) to induce T cell apoptosis
278 (*Mahoney, Freeman & McDermott, 2015; Zhang et al., 2016*). In this study, the proportions of
279 both CD8+ and CD4+ PD1+ T cells were increased in the renal allograft recipients, suggesting
280 that more reactive T cells were potentially undergoing apoptosis and reversible exhaustion in the
281 immune-stable allograft recipients. Additionally, the terminal effector stages of T cell
282 differentiation are indicated by upregulation of CD57 (effector phenotype) and PD1
283 (coinhibitory molecule, exhausted phenotype) expression(*Booiman et al., 2017*), which basically
284 is consistent with the above results.
285 As secondary signals, the T cell costimulatory molecules CD27 and CD28 play very pivotal roles
286 in full T cell activation (*Tanaskovic et al., 2017*). Many immune anergy and suppression
287 therapeutic strategies have focused on these molecules. In our study, CD27+CD28+
288 (costimulatory molecule double-positive) T cells in the CD4+ and CD8+ groups were reduced in
289 the renal allograft recipients, whereas CD27-CD28- (double-negative) T cells were increased.
290 Moreover, CD4+CD27+CD28+ T cells were decreased but CD4+CD27-CD28- T cells were
291 increased in the 1-year group patients compared with those of the 5-year group patients,
292 indicating that T cell costimulatory signals were reduced in the immune-stable allograft patients
293 in the short term. This result might be consistent with a recent study showing that immunological
294 aging-related expansion of highly differentiated CD28- T cells was associated with higher
295 immunosuppression (*Dedeoglu et al., 2016*).

296 The traditional T cell subpopulations of naive, CM (CCR7+CD45RA-), EM (CCR7- CD45RA-)
297 and effector T cells were first defined based on CCR7 and CD45RA expression (*Maecker,*

298   *McCoy & Nussenblatt, 2012*). Compared with those of naive T cells, memory T cells require
299   lower activation conditions and can rapidly induce alloimmune responses through synthesis of a
300   variety of inflammatory cytokines and cytolytic effectors (*Adams et al., 2003*). CD4+ EM T cells
301   are linked to the occurrence of acute cellular and antibody-mediated rejection (*Danger, Sawitzki*
302   *& Brouard, 2016*). Despite taking immunosuppressive drugs, renal allograft recipients still show
303   a danger of acute cellular and antibody-mediated rejection. Therefore, in our study, an increased
304   fraction of CD4+ EM T cells was noted in the renal allograft recipients compared with that of the
305   healthy volunteers. Additionally, CD8+ CM T cells were decreased in the renal allograft
306   recipients in our study. Given that investigations into the role of CCR7 in transplant processes
307   have yielded conflicting results (*Ziegler et al., 2006*), explaining the exact mechanism of this
308   phenomenon is difficult. Dedeoglu and his colleagues also investigated these memory T subsets
309   in both the peripheral blood and lymph nodes (LNs). They found that the median frequencies of
310   CD4+ EM and CD4+CD28null T cells were significantly higher within patients with allograft
311   rejection, but no other significant differences were observed for the other CD4+ and CD8+ T cell
312   subsets (*Dedeoglu et al., 2017*). More functional studies should focus on this subset in transplant
313   patients. Additionally, highly differentiated memory T cells are characterized by loss of the
314   costimulatory molecule CD28, making them less dependent on costimulation to become
315   activated (*Weng, Akbar & Goronzy, 2009*). Therefore, we also examined terminally
316   differentiated T cells. We analyzed the percentages of CM and EM cells in the CD28-positive
317   and -negative cell populations. We found that the frequency of CD4+CD28+EM cells in the
318   renal recipients was significantly higher and the frequency of CD8+CD28-CM cells was
319   significantly lower than those in the healthy volunteers (Supplementary Fig. 2). These
320   differences were consistent with the CD4+ EM and CD8+ CM cell results described above.

321   Interestingly, we found that the CD8+ T cells were expressed at low levels in our study, which
322   might have been the result of recent TCR stimulation, as indicated by the downregulation of CD8.
323   Therefore, we analyzed the CD8low subsets to observe whether they varied between the healthy
324   volunteer and patient groups. We found that the frequencies of CD57+ and CD27-CD28- subsets
325   from the CD8low T cells were significantly higher in the patient groups than in the healthy
326   volunteers. However, the frequencies of the PD1+, CD27+CD28+, CM and EM subsets from the
327   CD8low T cells were significantly lower in the patient groups than in the healthy volunteers

328   (Supplementary Fig. 3). The results were not completely consistent with those obtained for the

329   total CD8+ T subsets.

330   Renal failure and dialysis are known to change a patient's immune profile, which leads to T cell

331   dysfunction (*Betjes, 2013*). Although renal function in the enrolled subjects was corrected with

332   transplantation, whether the transplant also reverted the immune dysregulation was unclear.

333   Therefore, elucidating the impact of previous ESRD on the differences is important and

334   necessary. We enrolled uremia patients aged from 18-65 years who had undergone dialysis in

335   our department. None of these patients had received immunosuppressive treatment. Some useful

336   information and results were found compared with those of the healthy volunteers and renal

337   allograft recipients. 1) The frequency of CD8+ T cells was significantly lower than those in the

338   other groups, which was consistent with the description of Costa's and Cheng's studies (*Cheng,*

339   *Chen & Li, 1991; Costa et al., 2008*). 2) The frequency of the CD3+ HLA-DR+ T cell

340   population (activated T cells) in the uremia patients was significantly lower than that in the

341   healthy volunteers, and the frequencies of both the 1-year and 5-year recipients were also

342   significantly lower than that of the uremia patients. 3) In the TCR αβ and γδ subgroups, the

343   frequency of αβ T cells in the uremia patients was significantly higher and the frequency of γδ T

344   cells was significantly lower than those in the other groups. In a recent study, significant

345   inhibition of the γδ T cell population was demonstrated in patients with ESRD (*Juno et al., 2017*).

346   4) The frequencies of both CD4+ and CD8+ CM cells were significantly higher but the

347   frequencies of both CD4+ and CD8+ EM cells were significantly lower than those of the other

348   groups (Supplementary Fig. 4). A previous study showed that the percentages of CM and EM

349   T cells were significantly higher in the ESRD group than in the healthy group (*Chung et al.,*

350   *2012*). However, in our study, both CD4+ and CD8+ EM T cells showed a lower frequency in

351   the ESRD group. Although this finding was not consistent with Chung's observation, the ratio of

352   EM/CM T cells was decreased in the ESRD group, which was identical to the conclusion of

353   Segundo's study (*Segundo et al., 2010*). Taken together, an impact of previous renal disease and

354   dialysis indeed existed in some T cell subsets. However, we could also speculate, albeit not

355   strongly, that kidney transplantation might revert not only renal function but also T cell immune

356   dysregulation.

357 As stated in the beginning, most renal allograft recipients reach a stable immune state (neither
358 rejection nor infection) after transplantation. The importance of this study is that we provide an
359 overview of the stable immune state of renal allograft recipients. We evaluated the immune state
360 of renal allograft recipients based on normal immunity in the past, but renal allograft recipients
361 also have their own stable immune state. We wanted to identify differences between this stable
362 immune state and a normal immune state. In the future, we may perform a large sample-size
363 study to provide the basic background and criteria for renal allograft recipient immunity and
364 conduct further studies to evaluate immune cell changes in response to infection, rejection and
365 other states according to stable immunity instead of normal immunity. For this purpose, we
366 should elucidate the differences, and thus a large sample size study may be warranted to define
367 the criteria.

368

369 **CONCLUSION**

370 We comprehensively evaluated the immune state of stable renal allograft recipients in detail and
371 found that the distribution of most T cell subpopulations in immune-stable renal allograft
372 recipients significantly differed from those of healthy volunteers, including γδ, Vδ2, PD1,
373 CD27+ CD28+, CD27-CD28-, CD4+ EM and CD8+ CM cells. The proportion of some of these
374 T cell subsets in the renal allograft recipients also differed in the short-term compared with those
375 in the long-term. However, more detailed information should be included in subsequent studies,
376 such as the absolute numbers of each T cell subset and the proportion of regulatory T cells.

377

378

379 **Acknowledgements**

380 Not applicable.

381

382 **Data Availability**

383 The following information was supplied regarding data availability: The raw data and
384 supplementary figures are provided in the Supplemental Files.

385

386 **Consent for publication**

387   Not applicable.

388

389   **REFERENCES**

390   **Adams, A. B., Williams, M. A., Jones, T. R., Shirasugi, N., Durham, M. M., Kaech, S. M.,**
391   **Wherry, E. J., Onami, T., Lanier, J. G., Kokko, K. E., Pearson, T. C., Ahmed, R.,**
392   **Larsen, C. P. 2003.** Heterologous immunity provides a potent barrier to transplantation
393   tolerance. *J Clin Invest,* **111:** 1887-1895 DOI 10.1172/JCI17477.

394   **Agarwal, A., Newell, K. A. 2008.** The role of positive costimulatory molecules in
395   transplantation and tolerance. *Curr Opin Organ Transplant,* **13:** 366-372 DOI
396   10.1097/MOT.0b013e328306115b.

397   **Arneth, B. M. 2018.** Activation of CD4 and CD8 T cell receptors and regulatory T cells in
398   response to human proteins. *PeerJ,* **6:** e4462 DOI 10.7717/peerj.4462.

399   **Bansal, R. R., Mackay, C. R., Moser, B., Eberl, M. 2012.** IL-21 enhances the potential of
400   human gammadelta T cells to provide B-cell help. *Eur J Immunol,* **42:** 110-119 DOI
401   10.1002/eji.201142017.

402   **Betjes, M. G. 2013.** Immune cell dysfunction and inflammation in end-stage renal disease. *Nat*
403   *Rev Nephrol,* **9:** 255-265 DOI 10.1038/nrneph.2013.44.

404   **Booiman, T., Wit, F. W., Girigorie, A. F., Maurer, I., De Francesco, D., Sabin, C. A.,**
405   **Harskamp, A. M., Prins, M., Franceschi, C., Deeks, S. G., Winston, A., Reiss, P.,**
406   **Kootstra, N. A., Co-morBidity in Relation to Aids, C. 2017.** Terminal differentiation
407   of T cells is strongly associated with CMV infection and increased in HIV-positive
408   individuals on ART and lifestyle matched controls. *PLoS One,* **12:** e0183357 DOI
409   10.1371/journal.pone.0183357.

410   **Brandes, M., Willimann, K., Moser, B. 2005.** Professional antigen-presentation function by
411   human gammadelta T Cells. *Science,* **309:** 264-268 DOI 10.1126/science.1110267.

412   **Brenchley, J. M., Karandikar, N. J., Betts, M. R., Ambrozak, D. R., Hill, B. J., Crotty, L. E.,**
413   **Casazza, J. P., Kuruppu, J., Migueles, S. A., Connors, M., Roederer, M., Douek, D.**
414   **C., Koup, R. A. 2003.** Expression of CD57 defines replicative senescence and antigen-
415   induced apoptotic death of CD8+ T cells. *Blood,* **101:** 2711-2720 DOI 10.1182/blood-
416   2002-07-2103.

417   **Busch, D. H., Frassle, S. P., Sommermeyer, D., Buchholz, V. R., Riddell, S. R. 2016.** Role of

418   memory T cell subsets for adoptive immunotherapy. *Semin Immunol,* **28:** 28-34 DOI

419   10.1016/j.smim.2016.02.001.

420   **Caccamo, N., La Mendola, C., Orlando, V., Meraviglia, S., Todaro, M., Stassi, G., Sireci, G.,**

421   **Fournie, J. J., Dieli, F. 2011.** Differentiation, phenotype, and function of interleukin-17-

422   producing human Vgamma9Vdelta2 T cells. *Blood,* **118:** 129-138 DOI 10.1182/blood-

423   2011-01-331298.

424   **Cheng, Q. L., Chen, X. M., Li, L. S. 1991.** [Analysis of lymphocyte subpopulation in chronic

425   dialyzed end stage renal failure by flow cytometry]. *Zhonghua Nei Ke Za Zhi,* **30:** 506-

426   508, 522 DOI

427   **Chung, B. H., Kim, K. W., Sun, I. O., Choi, S. R., Park, H. S., Jeon, E. J., Kim, B. M., Choi,**

428   **B. S., Park, C. W., Kim, Y. S., Cho, M. L., Yang, C. W. 2012.** Increased interleukin-17

429   producing effector memory T cells in the end-stage renal disease patients. *Immunol Lett,*

430   **141:** 181-189 DOI 10.1016/j.imlet.2011.10.002.

431   **Costa, E., Lima, M., Alves, J. M., Rocha, S., Rocha-Pereira, P., Castro, E., Miranda, V., do,**

432   **S. F., Loureiro, A., Quintanilha, A., Belo, L., Santos-Silva, A. 2008.** Inflammation, T-

433   cell phenotype, and inflammatory cytokines in chronic kidney disease patients under

434   hemodialysis and its relationship to resistance to recombinant human erythropoietin

435   therapy. *J Clin Immunol,* **28:** 268-275 DOI 10.1007/s10875-007-9168-x.

436   **Croft, M. 2003.** Co-stimulatory members of the TNFR family: keys to effective T-cell immunity?

437   *Nat Rev Immunol,* **3:** 609-620 DOI 10.1038/nri1148.

438   **Cura Daball, P., Ventura Ferreira, M. S., Ammann, S., Klemann, C., Lorenz, M. R.,**

439   **Warthorst, U., Leahy, T. R., Conlon, N., Roche, J., Soler-Palacin, P., Garcia-Prat,**

440   **M., Fuchs, I., Fuchs, S., Beier, F., Brummendorf, T. H., Speckmann, C., Olbrich, P.,**

441   **Neth, O., Schwarz, K., Ehl, S., Rensing-Ehl, A. 2018.** CD57 identifies T cells with

442   functional senescence before terminal differentiation and relative telomere shortening in

443   patients with activated PI3 kinase delta syndrome. *Immunol Cell Biol* DOI

444   10.1111/imcb.12169.

445   **Danger, R., Sawitzki, B., Brouard, S. 2016.** Immune monitoring in renal transplantation: The

446   search for biomarkers. *Eur J Immunol,* **46:** 2695-2704 DOI 10.1002/eji.201545963.

447   **Darres, A., Ulloa, C., Brakemeier, S., Garrouste, C., Bestard, O., Del Bello, A., Sberro**
448   **Soussan, R., Durr, M., Budde, K., Legendre, C., Kamar, N. 2018.** Conversion to
449   belatacept in maintenance kidney-transplant patients: A retrospective multicenter
450   European study.   *Transplantation* DOI 10.1097/TP.0000000000002192.
451   **Dedeoglu, B., Litjens, N. H. R., de Weerd, A. E., Dor, F. J., Klepper, M., Reijerkerk, D.,**
452   **Baan, C. C., Betjes, M. G. H. 2017.** T-Cell Composition of the Lymph Node Is
453   Associated with the Risk for Early Rejection after Renal Transplantation.   *Front*
454   *Immunol,* **8:** 1416 DOI 10.3389/fimmu.2017.01416.
455   **Dedeoglu, B., Meijers, R. W., Klepper, M., Hesselink, D. A., Baan, C. C., Litjens, N. H.,**
456   **Betjes, M. G. 2016.** Loss of CD28 on Peripheral T Cells Decreases the Risk for Early
457   Acute Rejection after Kidney Transplantation.   *PLoS One,* **11:** e0150826 DOI
458   10.1371/journal.pone.0150826.
459   **Ferreira, V. H., Kumar, D., Humar, A. 2018.** Deep Profiling of the CD8+ T cell Compartment
460   identifies Activated cell subsets and Multifunctional Responses Associated with Control
461   of Cytomegalovirus Viremia.   *Transplantation* DOI 10.1097/TP.0000000000002373.
462   **Frassanito, M. A., Silvestris, F., Cafforio, P., Dammacco, F. 1998.** CD8+/CD57 cells and
463   apoptosis suppress T-cell functions in multiple myeloma.   *Br J Haematol,* **100:** 469-477.
464   **Goldschmidt, I., Karch, A., Mikolajczyk, R., Mutschler, F., Junge, N., Pfister, E. D.,**
465   **Mohring, T., d'Antiga, L., McKiernan, P., Kelly, D., Debray, D., McLin, V.,**
466   **Pawlowska, J., Hierro, L., Daemen, K., Keil, J., Falk, C., Baumann, U. 2018.**
467   Immune monitoring after pediatric liver transplantation - the prospective ChilSFree
468   cohort study.   *BMC Gastroenterol,* **18:** 63 DOI 10.1186/s12876-018-0795-x.
469   **Hartono, C., Muthukumar, T., Suthanthiran, M. 2013.** Immunosuppressive drug therapy.
470   *Cold Spring Harb Perspect Med,* **3:** a015487 DOI 10.1101/cshperspect.a015487.
471   **Juno, J. A., Waruk, J. L. M., Harris, A., Mesa, C., Lopez, C., Bueti, J., Ball, T. B., Kiazyk,**
472   **S. A. 2017.** gammadelta T-cell function is inhibited in end-stage renal disease and
473   impacted by latent tuberculosis infection.   *Kidney Int,* **92:** 1003-1014 DOI
474   10.1016/j.kint.2017.03.036.
475   **Kared, H., Martelli, S., Ng, T. P., Pender, S. L., Larbi, A. 2016.** CD57 in human natural killer
476   cells and T-lymphocytes.   *Cancer Immunol Immunother,* **65:** 441-452 DOI

477   10.1007/s00262-016-1803-z.

478 **Larbi, A., Fulop, T. 2014.** From "truly naive" to "exhausted senescent" T cells: when markers
479   predict functionality.   *Cytometry A,* **85:** 25-35 DOI 10.1002/cyto.a.22351.

480 **Maecker, H. T., McCoy, J. P., Nussenblatt, R. 2012.** Standardizing immunophenotyping for
481   the Human Immunology Project.   *Nat Rev Immunol,* **12:** 191-200 DOI 10.1038/nri3158.

482 **Mahoney, K. M., Freeman, G. J., McDermott, D. F. 2015.** The Next Immune-Checkpoint
483   Inhibitors: PD-1/PD-L1 Blockade in Melanoma.   *Clin Ther,* **37:** 764-782 DOI
484   10.1016/j.clinthera.2015.02.018.

485 **Mota, A. P., Vilaca, S. S., das Merces, F. L., Jr., Pinheiro Mde, B., Teixeira-Carvalho, A.,**
486   **Silveira, A. C., Martins-Filho, O. A., Gomes, K. B., Dusse, L. M. 2013.** Cytokines
487   signatures in short and long-term stable renal transplanted patients.   *Cytokine,* **62:** 302-
488   309 DOI 10.1016/j.cyto.2013.03.001.

489 **Perez-Gutierrez, A., Metes, D. M., Lu, L., Hariharan, S., Thomson, A. W., Ezzelarab, M. B.**
490   **2018.** Characterization of eomesodermin and T-bet expression by allostimulated CD8(+)
491   T cells of healthy volunteers and kidney transplant patients in relation to graft outcome.
492   *Clin Exp Immunol,* **194:** 259-272 DOI 10.1111/cei.13162.

493 **Peters, C., Hasler, R., Wesch, D., Kabelitz, D. 2016.** Human Vdelta2 T cells are a major source
494   of interleukin-9.   *Proc Natl Acad Sci U S A,* **113:** 12520-12525 DOI
495   10.1073/pnas.1607136113.

496 **Peters, C., Kabelitz, D., Wesch, D. 2018.** Regulatory functions of gammadelta T cells.   *Cell*
497   *Mol Life Sci* DOI 10.1007/s00018-018-2788-x.

498 **Ruhle, P. F., Fietkau, R., Gaipl, U. S., Frey, B. 2016.** Development of a Modular Assay for
499   Detailed Immunophenotyping of Peripheral Human Whole Blood Samples by Multicolor
500   Flow Cytometry.   *Int J Mol Sci,* **17** DOI 10.3390/ijms17081316.

501 **Sadat-Sowti, B., Debre, P., Mollet, L., Quint, L., Hadida, F., Leblond, V., Bismuth, G.,**
502   **Autran, B. 1994.** An inhibitor of cytotoxic functions produced by CD8+CD57+ T
503   lymphocytes from patients suffering from AIDS and immunosuppressed bone marrow
504   recipients.   *Eur J Immunol,* **24:** 2882-2888 DOI 10.1002/eji.1830241145.

505 **Sallusto, F., Lenig, D., Forster, R., Lipp, M., Lanzavecchia, A. 1999.** Two subsets of memory
506   T lymphocytes with distinct homing potentials and effector functions.   *Nature,* **401:**

507   708-712 DOI 10.1038/44385.

508   **Segundo, D. S., Fernandez-Fresnedo, G., Gago, M., Beares, I., Ruiz-Criado, J., Gonzalez,**
509   **M., Ruiz, J. C., Gomez-Alamillo, C., Lopez-Hoyos, M., Arias, M. 2010.** Kidney
510   transplant recipients show an increase in the ratio of T-cell effector memory/central
511   memory as compared to nontransplant recipients on the waiting list. *Transplant Proc,*
512   **42:** 2877-2879 DOI 10.1016/j.transproceed.2010.07.072.

513   **Siu, J. H. Y., Surendrakumar, V., Richards, J. A., Pettigrew, G. J. 2018.** T cell
514   Allorecognition Pathways in Solid Organ Transplantation. *Front Immunol,* **9:** 2548
515   DOI 10.3389/fimmu.2018.02548.

516   **Streitz, M., Miloud, T., Kapinsky, M., Reed, M. R., Magari, R., Geissler, E. K., Hutchinson,**
517   **J. A., Vogt, K., Schlickeiser, S., Kverneland, A. H., Meisel, C., Volk, H. D., Sawitzki,**
518   **B. 2013.** Standardization of whole blood immune phenotype monitoring for clinical trials:
519   panels and methods from the ONE study. *Transplant Res,* **2:** 17 DOI 10.1186/2047-
520   1440-2-17.

521   **Strioga, M., Pasukoniene, V., Characiejus, D. 2011.** CD8+ CD28- and CD8+ CD57+ T cells
522   and their role in health and disease. *Immunology,* **134:** 17-32 DOI 10.1111/j.1365-
523   2567.2011.03470.x.

524   **Tanaskovic, S., Price, P., French, M. A., Fernandez, S. 2017.** Impaired Upregulation of the
525   Costimulatory Molecules, CD27 and CD28, on CD4(+) T Cells from HIV Patients
526   Receiving ART Is Associated with Poor Proliferative Responses. *AIDS Res Hum*
527   *Retroviruses,* **33:** 101-109 DOI 10.1089/AID.2015.0327.

528   **Tanko, R. F., Soares, A. P., Masson, L., Garrett, N. J., Samsunder, N., Abdool Karim, Q.,**
529   **Abdool Karim, S. S., Riou, C., Burgers, W. A. 2018.** Residual T cell activation and
530   skewed CD8+ T cell memory differentiation despite antiretroviral therapy-induced HIV
531   suppression. *Clin Immunol* DOI 10.1016/j.clim.2018.06.001.

532   **Tyler, C. J., Doherty, D. G., Moser, B., Eberl, M. 2015.** Human Vgamma9/Vdelta2 T cells:
533   Innate adaptors of the immune system. *Cell Immunol,* **296:** 10-21 DOI
534   10.1016/j.cellimm.2015.01.008.

535   **Wang, L., Han, R., Hancock, W. W. 2007.** Programmed cell death 1 (PD-1) and its ligand PD-
536   L1 are required for allograft tolerance. *Eur J Immunol,* **37:** 2983-2990 DOI

537   10.1002/eji.200737583.

538 **Weng, N. P., Akbar, A. N., Goronzy, J. 2009.** CD28(-) T cells: their role in the age-associated
539   decline of immune function. *Trends Immunol,* **30:** 306-312 DOI
540   10.1016/j.it.2009.03.013.

541 **Wesch, D., Glatzel, A., Kabelitz, D. 2001.** Differentiation of resting human peripheral blood
542   gamma delta T cells toward Th1- or Th2-phenotype. *Cell Immunol,* **212:** 110-117 DOI
543   10.1006/cimm.2001.1850.

544 **Xu, D., Robinson, A. P., Ishii, T., Duncan, D. S., Alden, T. D., Goings, G. E., Ifergan, I.,**
545   **Podojil, J. R., Penaloza-MacMaster, P., Kearney, J. A., Swanson, G. T., Miller, S. D.,**
546   **Koh, S. 2018.** Peripherally derived T regulatory and gammadelta T cells have opposing
547   roles in the pathogenesis of intractable pediatric epilepsy. *Journal of Experimental*
548   *Medicine,* **215:** 1169-1186 DOI 10.1084/jem.20171285.

549 **Yang, J., Brook, M. O., Carvalho-Gaspar, M., Zhang, J., Ramon, H. E., Sayegh, M. H.,**
550   **Wood, K. J., Turka, L. A., Jones, N. D. 2007.** Allograft rejection mediated by memory
551   T cells is resistant to regulation. *Proc Natl Acad Sci U S A,* **104:** 19954-19959 DOI
552   10.1073/pnas.0704397104.

553 **Yu, M., Liu, M., Zhang, W., Ming, Y. 2018.** Pharmacokinetics, Pharmacodynamics and
554   Pharmacogenetics of Tacrolimus in Kidney Transplantation. *Curr Drug Metab* DOI
555   10.2174/1389200219666180129151948.

556 **Zhang, J., Wang, C. M., Zhang, P., Wang, X., Chen, J., Yang, J., Lu, W., Zhou, W., Yuan,**
557   **W., Feng, Y. 2016.** Expression of programmed death 1 ligand 1 on periodontal tissue
558   cells as a possible protective feedback mechanism against periodontal tissue destruction.
559   *Mol Med Rep,* **13:** 2423-2430 DOI 10.3892/mmr.2016.4824.

560 **Ziegler, E., Gueler, F., Rong, S., Mengel, M., Witzke, O., Kribben, A., Haller, H.,**
561   **Kunzendorf, U., Krautwald, S. 2006.** CCL19-IgG prevents allograft rejection by
562   impairment of immune cell trafficking. *J Am Soc Nephrol,* **17:** 2521-2532 DOI
563   10.1681/ASN.2005070782.

564

Inclusion and exclusion criteria in our study.

CDC: complement-dependent cytotoxicity, WBC: white blood cell, Pred: prednisolone, DSA: donor specific antibody, HCV: hepatitis C virus, HBsAg: hepatitis B surface antigen.

1   **Table 1. Inclusion and exclusion criteria in our study.**

Inclusion criteria	Exclusion criteria
CDC test negative on the day of transplant	Existed DSA on the day of transplant
WBC count, total platelet count, and renal function within normal limits	Experienced proved bacterial and fungi infection at time of transplantation and (or) blood collection
Took FK506+MMF+Pred for their long-term maintenance immunosuppressant	Undertook organ transplantation previously
	Had a history of malignancy
	Serum positive for HIV, HCV antibody, or HBsAg (The latest result before transplantation)
	Pregnancy at time of blood collection
	Received a lymphocyte depleting therapy

2   CDC: complement-dependent cytotoxicity, WBC: white blood cell, Pred: prednisolone, DSA:

3   donor specific antibody, HCV: hepatitis C virus, HBsAg: hepatitis B surface antigen.

Overview of the 2 staining panels each dedicated to a specific cell type which is indicated by individual colors.

FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Phycoerythrin-Texas Red-X, PE-Cy: Phycoerythrin-Cyanine, APC: Allophycocyanin, AF700: Alexa Flour 700, AA750: Allophycocyanin Alexa Flour 750, PB: Pacific Blue, KRO: Krome Orange., CC: compensation control.

N

1 **Table 2. Overview of the 2 staining panels each dedicated to a specific cell type which is**

2 **indicated by individual colors.**

Excitation (nm)	Blue: 488					Red: 633			Violent: 405	
Emission (nm)	523	575	613	692	760	650	720	767	455	528
Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PE-Cy7	APC	AF700	AA750	PB	KRO
Panel 1	CD45RA	CCR7	CD28	PD1	CD27	CD4	CD8	CD3	CD57	CD45
CC of panel 1	CD4	CD4	CD28	PD1	CD27	CD4	CD8	CD3	CD4	CD8
Panel 2	TCRγδ	TCRαβ	HLA-DR	-	TCR Vδ1	CD4	CD8	CD3	TCR Vδ2	CD45
CC of panel 1	CD4	CD4	HLA-DR	-	TCR Vδ1	CD4	CD8	CD3	CD4	CD8

3 FITC: Fluorescein isothiocyanate, PE: Phycoerythrin, ECD: Phycoerythrin-Texas Red-X, PE-

4 Cy: Phycoerythrin-Cyanine, APC: Allophycocyanin, AF700: Alexa Flour 700, AA750:

5 Allophycocyanin Alexa Flour 750, PB: Pacific Blue, KRO: Krome Orange., CC: compensation

6 control.

Baseline data in different groups (Mean±SD)

1 **Table 3. Baseline data in different groups (Mean±SD)**

	1-year	5-year	Healthy	P value
Age, yrs (±SD)	35.81±8.69	44.53±10.02	38.11±9.55	<0.0001
Gender (M/F)	43/9	33/18	22/22	0.003
WBC (x10^9/L)	7.81±2.04	7.37±1.20	6.71±1.95	0.0111
Lymphocytes (x10^9/L)	2.02±0.69	2.15±0.57	1.26±0.43	<0.0001
Serum creatinine (umol/L)	117.4±26.76	101.88±25.94	71.13±17.53	0.0015
FK506 mean concentration (ng/mL)	7.16±1.74	5.87±1.54	-	0.0002

The mean, SD and P value of T subsets among healthy volunteers, 1-year and 5-year renal allograft recipients.

1 Table 4. The mean, SD and P value of T subsets among healthy volunteers, 1-year and 5-year

2 renal allograft recipients.

	Mean±SD			P value		
	Healthy (H)	1-year (1y)	5-year (5y)	H vs 1y	H vs 5y	1y vs 5y
CD3+	72.08±7.07	67.65±11.07	62.88±14.62	0.0302	0.0005	0.0796
CD4+	38.77±7.89	35.73±11.15	35.03±10.91	0.1484	0.0791	0.7687
CD8+	32.63±6.97	34.64±8.72	33.15±9.88	0.2410	0.7829	0.4559
CD3+HLA-DR+	55.38±19.77	51.37±11.91	49.28±16.09	0.2457	0.1291	0.4858
αβ	85.22±11.41	90.46±6.09	91.25±6.16	0.0076	0.0038	0.5474
γδ	14.20±11.39	8.99±5.82	8.04±6.04	0.0072	0.003	0.4577
Vδ1 γδ	17.28±17.87	35.47±16.22	35.81±23.13	<0.0001	0.0001	0.9354
Vδ2 γδ	73.96±22.10	43.96±22.13	46.52±28.61	<0.0001	<0.0001	0.6374
CD4+ CD57+	3.77±2.76	8.34±8.44	4.31±3.63	0.0014	0.4540	0.0057
CD4+ PD1+	32.36±9.09	38.47±12.87	38.60±15.89	0.0105	0.0271	0.9652
CD8+ CD57+	36.71±13.36	41.20±13.44	42.59±14.90	0.1212	0.0636	0.6453
CD8+ PD1+	25.60±8.81	34.17±13.50	32.34±13.25	0.0008	0.0082	0.9240
CD4+ CD28+CD27+	86.59±4.63	74.31±11.74	82.92±7.44	<0.0001	0.0088	0.0001
CD4+ CD28-CD27-	5.22±2.73	15.01±9.85	7.15±7.11	<0.0001	0.1073	<0.0001
CD8+ CD28+CD27+	50.02±13.34	36.21±12.61	34.53±14.48	<0.0001	<0.0001	0.5620
CD8+ CD28-CD27-	34.74±12.13	47.64±12.60	52.32±15.63	<0.0001	<0.0001	0.1238
CD4+ CM T	45.53±8.38	40.13±12.96	56.23±10.87	0.0250	<0.0001	<0.0001
CD4+ EM T	15.02±4.82	25.74±15.14	22.76±10.40	<0.0001	<0.0001	0.2933
CD8+ CM T	7.56±3.66	4.93±4.59	5.71±4.29	0.0043	0.0394	0.4157
CD8+ EM T	31.37±9.86	28.17±12.71	26.07±13.08	0.1945	0.0415	0.4489

Figure 1

Schematic overview and flow cytometric gating strategies of T cell subsets in peripheral blood.
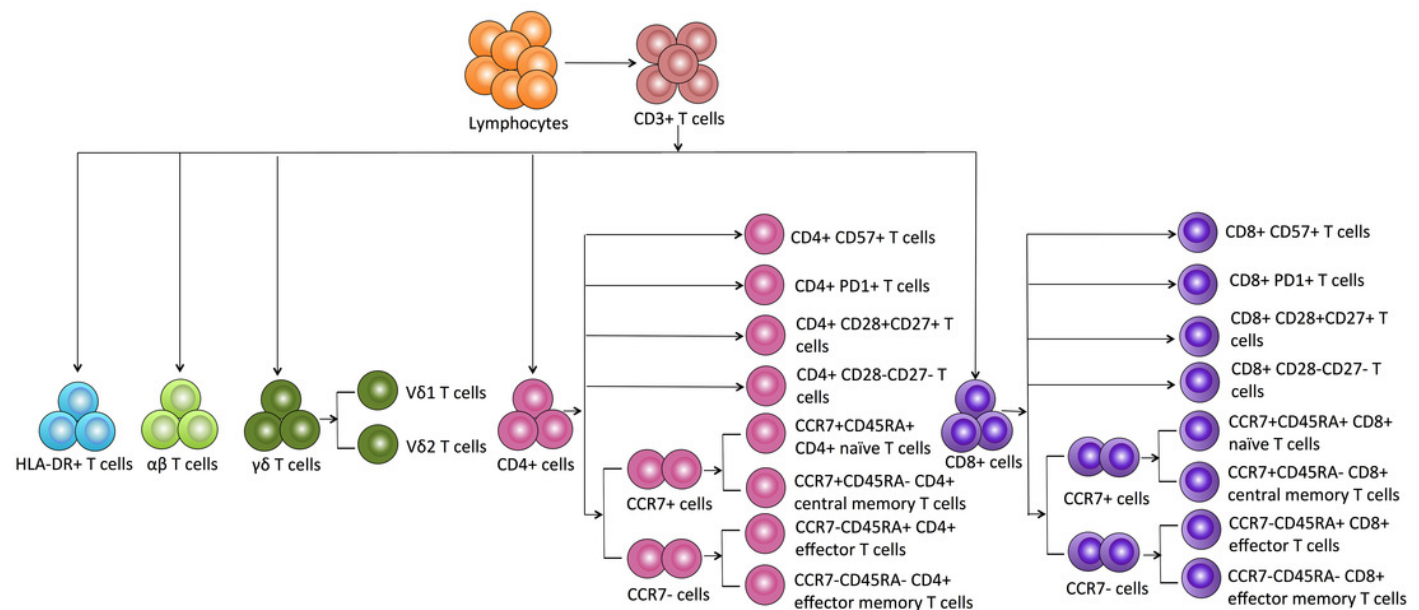
Figure 2

Parental proportions of total, CD4+, CD8+, HLA-DR+ T cells among different groups.

Healthy individuals showed a higher percentage of total (CD3+) T cells than both 1-year (p<0.05) and 5-year (p<0.001) renal allograft recipients (A and B). The differences of CD4+, CD8+, HLA-DR+ T cells were not significant (p>0.05) (C-F). Data are expressed as mean number of each group (mean±SD). *p<0.05, *** p<0.001.
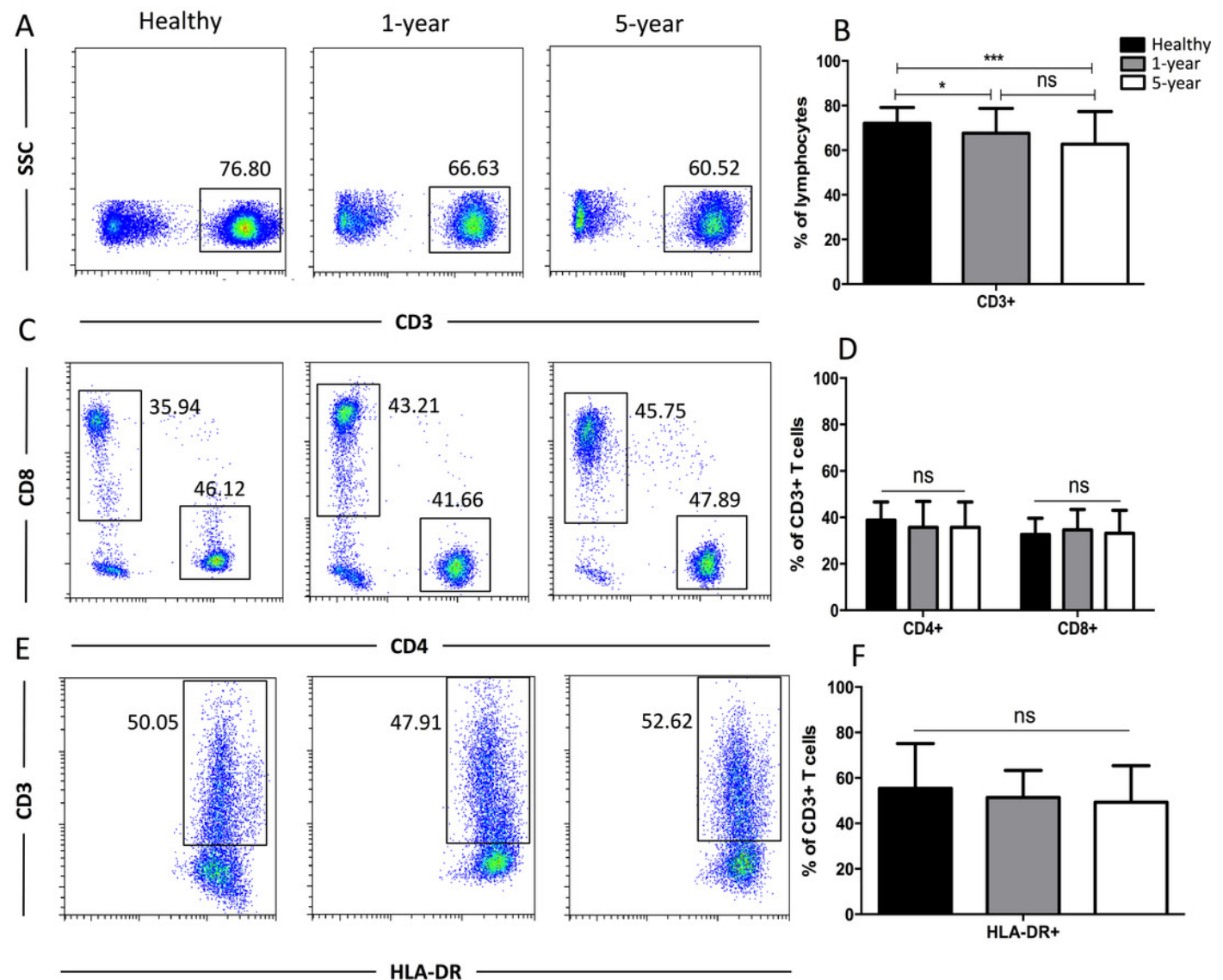
Figure 3

Parental proportions of αβ, γδ, and Vδ1 and Vδ2 γδ T cells among different groups.

Healthy individuals showed a lower percentage of αβ T cells, but a higher percentage of γδ T cells than both 1-year (p<0.01) and 5-year (p<0.01) renal allograft recipients (A and B). Healthy individuals also showed a lower percentage of Vδ1 but a higher percentage of Vδ2 γδ T cells than both 1-year (p<0.0001) and 5-year (p<0.0001) renal allograft recipients (C and D). The differences between 1-year and 5-year recipients from each TCR subsets above were not significant (p>0.05) (A-D). Data are expressed as mean number of each group (mean±SD). **p<0.01, ****p<0.0001.
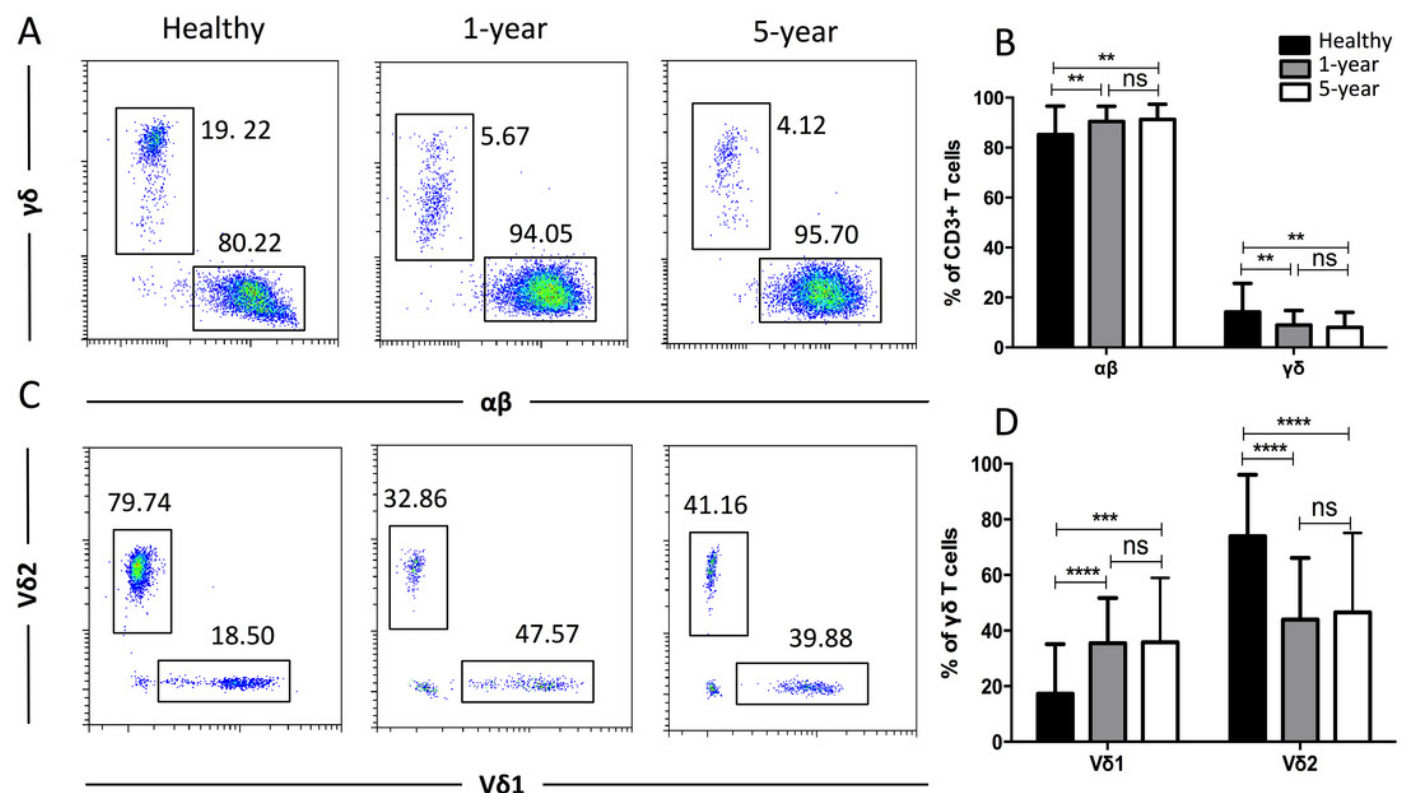
Figure 4

Parental proportions of CD57+ and PD1+ T cells among different groups.

In CD4+ T cells, the percentage of CD57+T cells was the highest in 1-year renal allograft recipients compared with healthy individuals (p<0.01) and 5-year recipients (p<0.01). No significant difference was addressed between healthy individuals and 5-year renal allograft patients (p>0.05). The percentage of PD1+T cells was significantly increased in renal allograft recipients than healthy individuals (p<0.05). No significant difference was addressed between 1-year and 5-year renal allograft patients (p>0.05) (A and B). In CD8+ T cells, no significant difference in CD57+ T cells was noted among all the 3 groups (p>0.05). The percentage of PD1+T cells populations was significantly increased in renal allograft recipients than healthy individuals (p<0.05). No significant difference was addressed between 1-year and 5-year renal allograft patients (p>0.05) (C and D). Data are expressed as mean number of each group (mean±SD). *p<0.05, **p<0.01.
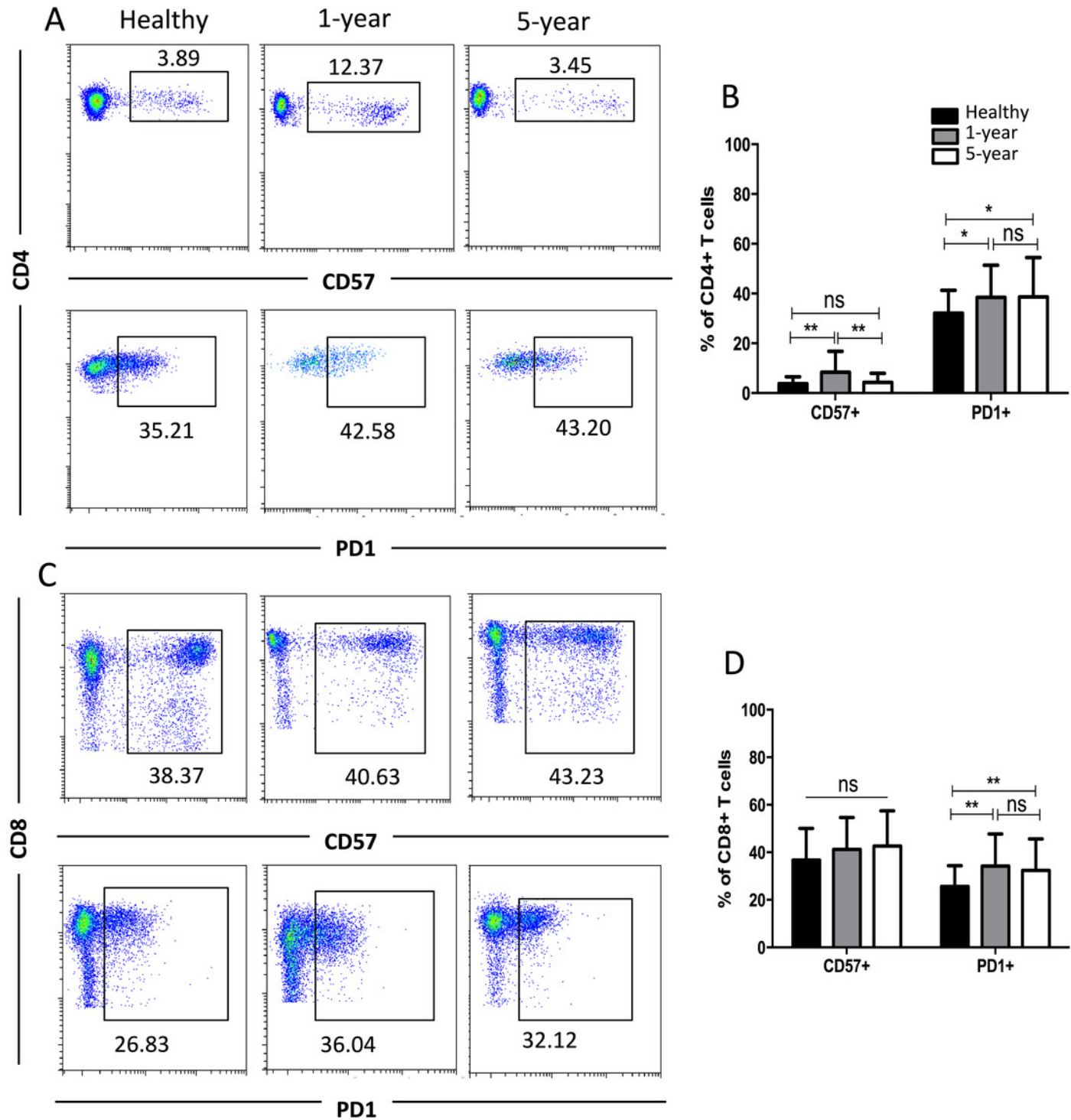
Figure 5

Parental proportions of costimulatory molecular (CD27 and CD28) T cells among different groups.

In CD4+ subsets, healthy individuals showed a higher percentage of CD27+CD28+ T cells than both 1-year (p<0.0001) and 5-year (p<0.01) renal allograft recipients; 1-year recipients had a lower percentage than 5-year group (p<0.001). Healthy individuals showed a lower percentage of CD27-CD28- T cells than both 1-year (p<0.0001) and 5-year (p<0.05) renal allograft recipients; 1-year recipients had a higher percentage than 5-year group (p<0.001) (A and B). In CD8+ subsets, healthy individuals showed a higher percentage of CD27+CD28+ T cells than both 1-year (p<0.0001) and 5-year (p<0.0001) renal allograft recipients. Healthy individuals showed a lower percentage of CD27-CD28- T cells than both 1-year (p<0.0001) and 5-year (p<0.0001) renal allograft recipients (C and D). The differences of both CD4+ and CD8+ CD27-CD28- T cells were not significant (p>0.05) (A-D). Data are expressed as mean number of each group (mean±SD). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.
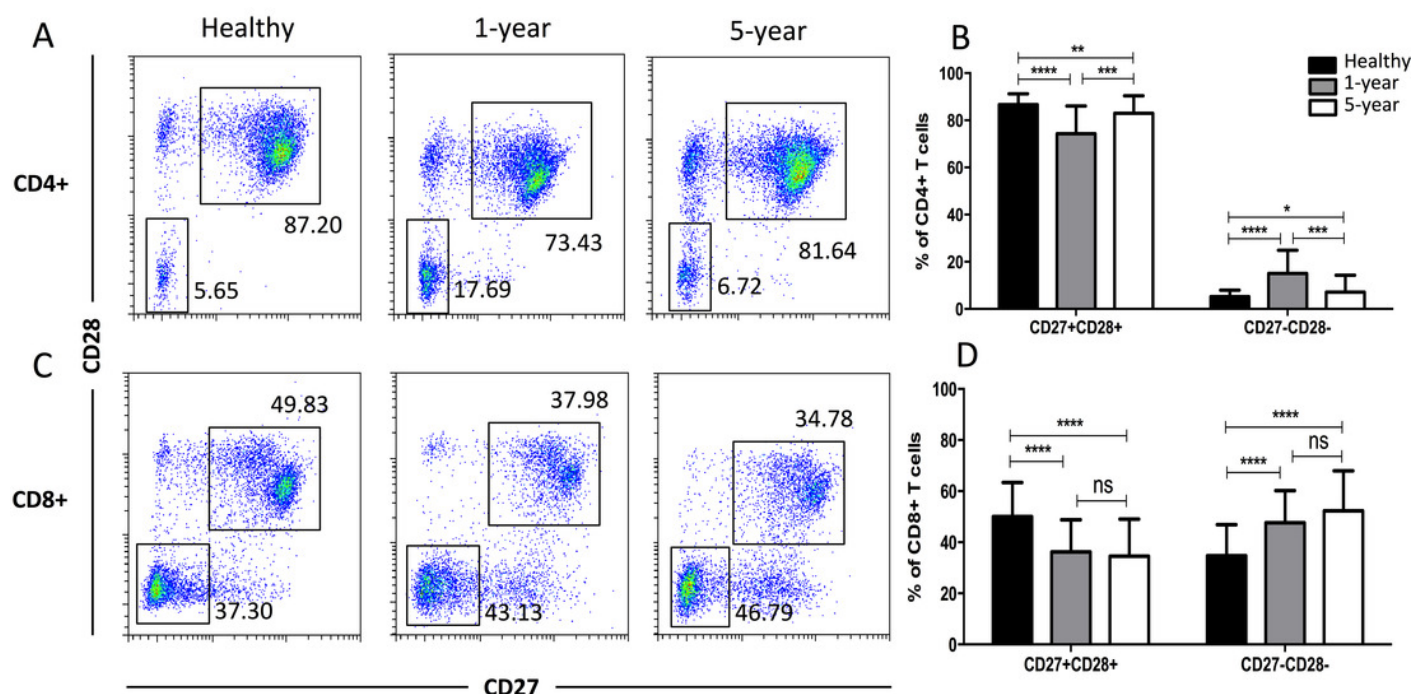
Figure 6

Parental proportions of memory T cells among different groups.

In CD4+ T cells, 5-year recipients showed a higher percentage of CM T cells than both 1-year (p<0.001) and healthy individuals (p<0.0001); 1-year recipients had a lower percentage than healthy individuals (p<0.05). Healthy individuals showed a higher percentage of EM T cells than both 1-year (p<0.0001) and 5-year (p<0.0001) renal allograft recipients,and no significant difference was addressed between 1-year and 5-year renal allograft patients (p>0.05) (A and B). In CD8+ T cells, healthy individuals showed a higher percentage of CM T cells than both 1-year (p<0.01) and 5-year (p<0.05) renal allograft recipients, and and no significant difference was addressed between 1-year and 5-year renal allograft patients (p>0.05). Healthy individuals showed a higher percentage of EM T cells than 5-year renal allograft recipients (p<0.05).The differences of CD8+ EM T cells were not significant both between healthy individuals and 1-year group and between 1-year and 5 year groups (p>0.05) (C and D). Data are expressed as mean number of each group (mean±SD). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.