1   ***TCF-7* is dispensable for anti-tumor response but required for**
2   **persistent function of mature CD8 T cells**
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Abstract

T Cell Factor-1, encoded by *TCF-7,* is a transcription factor that plays an essential role during T cell development and differentiation. In this manuscript we utilized a pre-clinical model provided evidence that *TCF-7* is dispensable for the anti-tumor response, and that *TCF-7* suppresses key transcriptional factors Eomes and T-bet and molecules responsible for peripheral CD8 T cell cytolytic function. We discovered that *TCF-7* regulates NKG2D expression on naïve and activated mouse CD8 T cells, and that peripheral CD8 T cells from *TCF-7* cKO utilize NKG2D to clear tumor cells.

We also provide evidence that *TCF-7* regulates key signaling molecules, including LCK, LAT, ITK, PLC-γ1, P65, ERKI/II, and JAK/STATs required for peripheral CD8 T cell persistent function. Our data transcriptomic and protein data uncovered the mechanism of how *TCF-7* impacting peripheral CD8 T cell inflammatory cytokine production, CD8 T cell activation, and apoptosis. Our pre-clinical model showed that CD8 T cells from *TCF-7* cKO mice did not cause GVHD, but effectively cleared primary tumor cells.

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Introduction

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65 T Cell Factor-1 (*TCF-7*), a major T cell developmental transcription factor, is involved in the

66 Wnt signaling pathway, and is critical for T cell development as well as activation (Escobar et

67 al., 2020; Ma et al., 2012). Dysfunction of the Wnt/β-catenin/*TCF-7* signaling pathway leads to

68 immune deficiency or autoimmunity (Shi et al., 2016). It is well known that *TCF-7* is involved in

69 the regulation of cell proliferation and survival during later T cell development (Kim et al.,

70 2020). In the absence of *TCF-7*, T cell development is completely blocked after early thymocyte

71 progenitor (ETP) cells, which suggests that *TCF-7* is also important in T cell lineage

72 specification (Germar et al., 2011; Weber et al., 2011). Several studies have shown that *TCF-7* is

73 critical for controlling viral infection (He et al., 2016; Im et al., 2016; Utzschneider et al., 2016).

74 Furthermore, *TCF-7* + CD8+ T cell have self-renewal capacity, while CD8+ T cells lacking

75 *TCF-7* do not (Utzschneider *et al.*, 2016; Wu et al., 2016). Overwhelming evidence has

76 suggested that *TCF-7* is critical for CD8 T cell persistence and capacity to control viral infection

77 (Kurtulus et al., 2019; Miller et al., 2019; Siddiqui et al., 2019; Wu *et al.*, 2016). While

78 investigating the role of *TCF-7* in viral infection, single cell RNA sequencing data uncovered

79 that CD8 T cells expressing higher levels of *TCF-7* are quiescent and tissue resident, while CD8

80 T cells expressing higher levels of *TCF-7* in periphery are highly proliferative, and that *TCF-7*

81 negative T-bet^{hi} CD8 T cells are transitory effector cells. *TCF-7*$^-$ Eomes^{hi} CD8 T cells are not

82 proliferative and express higher levels of immune checkpoint receptors, but maintain effector

83 function (Beltra et al., 2020; LaFleur et al., 2019; Zander et al., 2019). Studies have also reported

84 that *TCF-7*+ CD8 T cells with stem-like abilities expresses low levels of PD-1 and Tim

85 checkpoint receptors, further providing evidence that *TCF-7* is required for CD8 T cell formation

86 and persistent function (Kurtulus *et al.*, 2019; Siddiqui *et al.*, 2019; Utzschneider *et al.*, 2016).

87 Studies have demonstrated that dysfunctional virus-specific CD8 T cells transplanted into naïve

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88   mice give rise to *TCF-7*-negative CD8 T cells (Utzschneider et al., 2013). However, tumor-

89   specific CD8 T cells transferred to naïve mice might give rise to *TCF-7*+ CD8 T cells,

90   demonstrating the differential role of *TCF-7* in viral infection and tumor (Schietinger et al.,

91   2016). These differences could be due to the differences between microenvironments in viral

92   infection and in tumors (Philip et al., 2017). Whether *TCF-7*+ cells will give rise to *TCF-7*+ or

93   *TCF-7*- cells will be dependent on internal and external signaling. Some studies have shown that

94   both the long (p45) and the short (p33) *TCF-7* isoform are expressed by CD8 T cells that will

95   give rise to stem-like CD8 T cells during viral infection, and it has been shown that the long

96   isoform of *TCF-7* is capable of promoting stem-like CD8 T cell formation during viral infection

97   by regulating genes like CD127, CXCR5 and cMyb (Chen et al., 2019). Currently, the role of

98   long and short *TCF-7* isoforms is largely unknown.

99   Studies of T cells as immunotherapy in both human and mice showed that for a superior

100   anti-tumor response, less differentiated cells are more favorable than a more differentiated subset

101   of CD8 T cells (Gattinoni et al., 2011; Im *et al.*, 2016; Lugli et al., 2013). Ideal CD8 T cells for

102   immunotherapy have been shown to exhibit stem-like abilities (such as those obtained by

103   inducing *ex vivo* cell growth with IL-17, IL-15, IL-21) and higher expression of *TCF-7*, Eomes,

104   and Bcl6 (Cieri et al., 2013; Cui et al., 2011). Thus, the suitability of *TCF-7* as a target for

105   immunotherapy to clear viral infection and cancer might also indicate considerable consequences

106   for autoimmune diseases.

107   To study the role of *TCF-7* in a clinically relevant model, we utilized allogeneic

108   hematopoietic stem cell transplantation (allo-HSCT). In allo-HSCT, mature peripheral donor T

109   cells found in the graft become alloactivated upon recognition of host HLA as non-self. These T

110   cell activities help to clear residual malignant cells, which is called the graft-versus-leukemia

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111   effect (GVL) (Balassa et al., 2019; Giralt and Bishop, 2009; Hall and Shenoy, 2019). On the

112   other hand, alloactivated T cells also target healthy recipient tissues, an effect known as graft-

113   versus-host disease (GVHD) (Mavers and Bertaina, 2018). We used a unique mouse strain which

114   has deletion of *TCF-7* in mature T cells, rather than a global deletion (Xing et al., 2016). This

115   *TCF-7* flox/flox x CD4cre mouse experiences deletion of *TCF-7* in all T cells at the double-

116   positive phase of development, when all T cells express CD4 (Wang et al., 2019b). This allows

117   us to overcome the severe T cell developmental defect that occurs in global *TCF-7* deletion, as

118   *TCF-7* is critical for the double-negative stage of development (Yang et al., 2019).

119   Using a mouse model of GVHD and GVL following allo-HSCT, we were able to study all

120   of the major T cell functions, as well as phenotype, clinical outcomes, and gene expression, in a

121   single model. In this model, we transplanted CD8 T cells from either WT or *TCF-7* cKO mice

122   into irradiated BALB/c mice (H2K^b→H2K^d) (Mammadli et al., 2021a; Mammadli et al., 2021c;

123   Mammadli et al., 2021d). Using allogenic pre-clinical model, we have shown that CD8 T cells

124   from *TCF-7* cKO effectively clear tumor cells without inducing GVHD by producing

125   significantly less inflammatory cytokines as proinflammatory cytokines are con sidered the

126   hallmark of allo-immunity (Ju et al., 2005; Seif et al., 2017). Our data also uncovered that CD8 T

127   cells from *TCF-7* cKO mice cause significantly less tissue damage in GVHD target organs

128   (Bleakley et al., 2012; Breems and Lowenberg, 2005). Molecular analysis showed that CD8 T

129   cells from *TCF-7* cKO mice significantly showed reduction in key molecules required for CD8 T

130   cell persistent function (Germar *et al.*, 2011; Giralt and Bishop, 2009; Gounari and Khazaie,

131   2022). CD8 T cells from *TCF-7* cKO mice exhibited innate memory-like phenotype by

132   upregulating CD122, CD44, and effector and central memory phenotypes in mature CD8 T cells

133   critical for GVHD development (Dutt et al., 2011; Huang et al., 2013). We also uncovered that

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134   CD8 T cells from *TCF-7* cKO mice significantly upregulated Eomes and T-bet, two downstream

135   transcription factors which are known to be involved in GVL (Mammadli *et al.*, 2021a; Weeks et

136   al., 2021; Zhou et al., 2010). Our data demonstrated that naïve CD8 T cells from *TCF-7* cKO

137   mice upregulated NK cell type 2 receptor (NKG2D). NKG2D, encoded by *Klrk1*, is an activating

138   cell surface receptor that is predominantly expressed on Natural killer cells (Larson et al., 2006;

139   Wensveen et al., 2018). While naïve human CD8$^+$ T cells express NKG2D, in mice CD8 T cells

140   only upregulate NKG2D upon activation (Hu et al., 2016; Maasho et al., 2005). Upregulation of

141   Granzyme B on CD8 T cells from *TCF-7* cKO mice was also observed. The loss of *TCF-7* also

142   led to upregulation of NK cell type 2 receptor (NKG2D). NKG2D, encoded by *Klrk1*, is an

143   activating cell surface receptor that is predominantly expressed on Natural killer cells (Larson *et*

144   *al.*, 2006; Wensveen *et al.*, 2018). NKG2D is abundantly present on all NK cells, NKT cells, and

145   subsets of γδ T cells (Stojanovic et al., 2018). While naïve human CD8$^+$ T cells express NKG2D,

146   in mice CD8 T cells only upregulate NKG2D upon activation (Hu *et al.*, 2016; Maasho *et al.*,

147   2005). Upregulation of Granzyme B on CD8 T cells from *TCF-7* cKO mice was also observed.

148   Our molecular and animal data were confirmed by transcriptomic analysis.

149   Altogether, our data demonstrate that loss of *TCF-7* in mature murine CD8 T cells

150   enhanced Eomes and T-bet expression and reduced TCR-signaling, resulting in less severe

151   GVHD. Our data demonstrated that *TCF-7*-deficient CD8 T cells utilized NKG2D receptors to

152   kill tumor targets. These findings will have a considerable impact on developing strategies to

153   uncouple GVHD from GVL, and for developing therapeutic interventions for T cell-driven

154   autoimmune disorders.

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156 **Results**

157 **Loss of *TCF-7* in donor CD8 T cells reduced severity and persistence of GVHD symptoms,**

158 **increased survival from lethal GVHD, and retained anti-tumor capabilities for the GVL**

159 **effect.** Most of the previous research on *TCF-7* utilized a global *TCF-7* knockout because the

160 primary focus was on *TCF-7's* role as a developmental factor (Gounari and Khazaie, 2022;

161 Weber *et al.*, 2011). However, we wanted to study the role of *TCF-7* in mature T cells. Global

162 loss of *TCF-7* results in minimal T cell production from the thymus, because *TCF-7* is critical for

163 DN stages of development (Johnson et al., 2018). Therefore, we obtained mice with a T cell-

164 specific knockout for *TCF-7* (Tcf7 flox/flox x CD4cre, called *TCF-7* cKO here (Xing *et al.*,

165 2016). This allowed us to study mature T cells that developed normally in the thymus, then lost

166 expression of *TCF-7* at the DP phase (Berga-Bolanos et al., 2015).

167 To determine whether *TCF-7* plays a role in mature alloactivated T cell regulation, which

168 is currently unknown, we used a mouse model of MHC-mismatched allo-HSCT leading to

169 GVHD and GVL. Briefly, BALB/c mice (MHC haplotype d) were lethally irradiated and

170 transplanted with wild-type (WT) bone marrow and C57Bl/6-background (MHC haplotype b)

171 donor CD8 T cells (Mammadli *et al.*, 2021c; Mammadli *et al.*, 2021d). The donor CD8 T cells

172 came from wild-type (WT), or *TCF-7*-deficient (*TCF-7* cKO) mice. Recipients were given

173 $1X10^6$ CD8 T cells and $10X10^6$ WT T cell-depleted bone marrow cells, as well as $2X10^5$

174 luciferase-expressing B-cell lymphoma (A-20) cells (Edinger et al., 2003a; Edinger et al., 2003b)

175 to assess GVL responses (Mammadli *et al.*, 2021a; Mammadli et al., 2021b; Mammadli *et al.*,

176 2021c; Mammadli *et al.*, 2021d). A20 cells are syngeneic to BALB/c mice and allogeneic to

177 C57BL/6 (B6) mice (Edinger *et al.*, 2003a; Edinger *et al.*, 2003b), meaning that the cells will not

178 be naturally rejected by the BALB/c hosts, but will be attacked by the transplanted donor T cells.

179 The MHC haplotype mismatch between host and donor cells drives alloactivation of donor T

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180   cells, which in turn causes GVHD and GVL effects (Hoffmann et al., 2002). To examine disease

181   severity, progression, and recipient mouse survival, recipient mice were weighed and given a

182   clinical score three times per week following transplant, until about day 70 **(Fig. 1A-D)**. The

183   mice were scored based on six factors: skin integrity, fur texture, posture, activity level, weight

184   loss, and diarrhea (Cooke et al., 1996). Since the A20 cells express luciferase (called A20 luc)

185   (Mammadli *et al.*, 2021c), allowing us to track them by injecting D-luciferin into the recipient

186   mice and imaging them with an *in vivo* bioluminescence scanner (IVIS 50), the mice were

187   scanned one time per week with IVIS 50 until the end of the experiment **(Fig.1A, 1E).**

188   We found that mice who received WT donor CD8 T cells had a rapid increase in GVHD

189   severity, with a high score being reached by day 14, suggesting severe GVHD **(Fig. 1B)**. This

190   high score was maintained, suggesting persistent disease, and reached a high peak score at day

191   40 when the recipient mice died of disease burden **(Fig. 1B-D)**. WT-transplanted mice lost

192   weight initially and were unable to regain much weight (**Fig.1C**). In contrast, mice given *TCF-7*

193   cKO CD8 T cells had much better survival, lower peak and average clinical scores, minimal

194   disease burden, and a gain in weight following the initial weight loss period **(Fig.1B-D).** In

195   addition, the clinical scores for *TCF-7* cKO CD8 T cells transplanted mice quickly reduced to

196   near-control levels following peak score at day 10 **(Fig. 1B)**, suggesting that disease does not

197   persist in these mice. Therefore, loss of *TCF-7* in donor T cells led to reduced GVHD severity

198   and persistence, with improved survival **(Fig.1D)**.

199   Regarding anti-tumor effects, we observed that over time, the group receiving only bone

200   marrow and tumor cells showed a large increase in tumor growth **(Fig. 1A, 1E)**, because no T

201   cells were present to control the tumor cells. In contrast, most mice given CD8 T cells from any

202   donor type along with the BM and A20 luc cells were able to clear the tumor cells by the end of

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203 the experiment **(Fig. 1A, 1E)**. The GVL effect was maintained even in *TCF-7* cKO-transplanted

204 mice **(Fig. 1A, 1E)**. Altogether, these data show that *TCF-7* is dispensable for GVL effects, but

205 critical for GVHD. Therefore, loss of *TCF-7* in donor T cells provides a clinically optimal

206 phenotype, where GVHD severity is reduced but beneficial GVL effects are maintained.

207

208 **Loss of *TCF-7* drives changes to mature CD8 T cell phenotype which are primarily cell-**

209 ***extrinsic***. It has been shown that loss of *TCF-7* in late stages of T cell development led to

210 impaired output of CD4 T cells, and redirection of CD4 T cells to a CD8 T cell fate(Steinke et

211 al., 2014). To determine whether loss of *TCF-7* affected mature donor T cell phenotype, we

212 performed flow cytometry phenotyping on CD8 T cells **(Fig. 2)**. First, we confirmed the loss of

213 *TCF-7* expression in *TCF-7* cKO mice by flow cytometry **(Fig.2A).** Next, we examined whether

214 the loss of *TCF-7* also altered Eomesodermin (Eomes) and T-box transcription factor 21 (T-bet)

215 expression, both of which are downstream of *TCF-7* (Chen *et al.*, 2019). Some reports have

216 claimed that Eomes is activated by *TCF-7* (meaning loss of *TCF-7* reduces Eomes expression)

217 (Paley and Wherry, 2010). However, in our model of conditional *TCF-7* deletion, we found that

218 *TCF-7* cKO CD8 T cells had increased expression of Eomes compared to WT CD8 T cells **(Fig.**

219 **2B)**. Other reports have claimed that T-bet may be activated or not affected by *TCF-7* (Ma *et al.*,

220 2012), but we found that loss of *TCF-7* led to increased T-bet expression in CD8 T cells **(Fig.**

221 **2C)**. This suggests that *TCF-7* normally suppresses the expression of Eomes and T-bet in mature

222 CD8 T cells.

223   Some reports have suggested that CD44^{hi} T cells do not cause GVHD or cause less severe

224 GVHD (Dutt *et al.*, 2011). Therefore, we wanted to examine CD8 T cells from *TCF-7* cKO mice

225 for activation markers like CD44 and CD122. Our data showed that CD8 T cells from *TCF-7*

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226   cKO mice exhibit increased expression of CD122 and CD44 **(Fig. 2D-E)**. Next, using CD62L

227   and CD44 markers, we identified three major memory subsets: central memory (CD44^{hi}

228   CD62L^{hi}), effector memory (CD44^{hi} CD62L^{low}), and naive (CD44^{low} CD62L^{hi}) cells **(Fig.2F)**.

229   *TCF-7* cKO mice showed increased central memory CD8 T cell subsets and decreased naive

230   CD8 T cells **(Fig. 2F)**. Thus, loss of *TCF-7* results in a more memory-skewed phenotype for

231   CD8 T cells. Some reports have suggested that memory T cells delay induction of GVHD (Dutt

232   *et al.*, 2011; Mammadli *et al.*, 2021c), so this phenotypic change may be beneficial (Nakajima et

233   al., 2021).

234   Changes to cell phenotype in a knock-out mouse may be cell-intrinsic (due directly to

235   gene deficiency within the cell) or cell-extrinsic (due to changes in the microenvironment from

236   gene deficiency) (Decman et al., 2010; Mammadli *et al.*, 2021d). To determine whether the

237   phenotypic effects we observed were cell-intrinsic or cell-extrinsic, we developed a chimeric

238   mouse model. Briefly, we mixed bone marrow from WT and *TCF-7* cKO mice at a 1:4

239   (WT:TCF) ratio for a total of 50X10^6 BM cells, then used this mixture to reconstitute lethally

240   irradiated Thy1.1 mice. We used a 1:4 ratio based on our previous published work (Mammadli et

241   al., 2020; Mammadli *et al.*, 2021d), to ensure survival of the KO cells (based on our initial

242   observations that *TCF-7* cKO T cells did not proliferate well in culture). At 9 weeks post-

243   transplant, blood was taken to ensure reconstitution and survival of both donor types in each

244   mouse. At 10 weeks, splenocytes were phenotyped by flow cytometry, with donor cells being

245   identified by H2K^b, CD45.1 (WT), and CD45.2 (*TCF-7* cKO) markers (Mammadli *et al.*, 2020).

246   First, we looked at the *TCF-7* expression in CD45.1+ (WT), and CD45.2+ (*TCF-7* cKO)

247   cells and confirmed that cells from *TCF-7* cKO mice did not express *TCF-7* in chimeric mice

248   **(Supp.Fig.1A).** We did see a statistically significant increase in T-bet expression in CD8 T cells

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249   from *TCF-7* cKO donor cells compared to WT donor cells in chimeric mice, when we performed

250   a t-test (data not shown). However, when we compared the T-bet expression in chimeric versus

251   naïve CD8 T cells, we observed that T-bet expression in CD8 T cells from *TCF-7* cKO donor

252   mice was reduced to near-WT levels from elevated levels **(Supp. Fig. 1B).** This suggests that the

253   increased expression of T-bet seen in *TCF-7* cKO CD8 T cells from naive mice is a cell-extrinsic

254   effect.   Interestingly, in the chimeric mice we observed that Eomes and CD122 expression levels

255   in WT CD8 T cells were significantly increased to near-*TCF-7* cKO levels, suggesting that the

256   increase in Eomes and CD122 expression in CD8 T cells from *TCF-7* cKO mice is primarily

257   cell-intrinsic **(Supp.Fig.1C-D).**

258   Next, we examined the expression of CD44 and central memory phenotype in chimeric

259   mice. We observed that while the frequencies of these subsets were lower in *TCF-7* cKO-derived

260   CD8 T cells compared to WT-derived CD8 T cells in the chimera (opposite of the trend observed

261   in naive mice), this was because the frequencies of CD44 and CM phenotype in WT cells was

262   enhanced to the levels expressed by *TCF-7* cKO cells from naive mice **(Supp. Fig. 1E-F).**

263   These results suggest that the effects of *TCF-7* deficiency on CD44 and CM phenotype

264   expression in naïve mice could be primarily cell-intrinsic, with cell-extrinsic elements as well.

265   Interestingly, effector memory phenotype in the chimeric mice we observed that levels in WT

266   CD8 T cells were significantly increased to near-*TCF-7* cKO levels, suggesting that the increase

267   in effector memory phenotype in CD8 T cells from *TCF-7* cKO mice is primarily cell-intrinsic

268   **(Supp. Fig. 1G).** Finally, the naïve CD8 T cell population in the chimera coming from *TCF-7*

269   cKO bone marrow was significantly increased compared to CD8 T cells from WT bone marrow

270   and compared to naïve *TCF-7* cKO mice (**Supp. Fig. 1H**). This suggests that the effect on naive

271   CD8 T cells in *TCF-7* cKO mice could be either cell-intrinsic or cell-extrinsic. Altogether, these

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272   data suggest that the phenotypic changes seen in *TCF-7* cKO may be primarily cell-intrinsic,

273   with some additional cell-extrinsic effects being present.

274

275   **Loss of *TCF-7* alters cytotoxic mediator production in mature CD8 T cells.** Our data

276   demonstrated that the loss of *TCF-7* increases Eomes and T-bet expression in mature CD8 T

277   cells (**Fig. 2B-C**). Considering that Eomes and T-bet have been reported to play a central role in

278   anti-tumor responses, we hypothesized that by upregulating Eomes and T-bet, CD8 T cells

279   lacking *TCF-7* can maintain cytotoxicity, and that *TCF-7* is not required for CD8 T cell-

280   mediated cytolytic function (Zhu et al., 2010). We anticipated that CD8 T cells from *TCF-7* cKO

281   mice may have attenuated TCR signaling, so we examined this and other activating receptors by

282   flow cytometry. It is also known that Eomes and T-bet overexpression increases NKG2D

283   expression in NK cells (Kiekens et al., 2021). Considering that loss of *TCF-7* in mature T cells

284   led to upregulation of Eomes and T-bet expression, we hypothesized that loss of *TCF-7* may also

285   lead to upregulation of NKG2D expression in CD8 T cells and enhance the anti-tumor response.

286   Natural killer group 2 member D (NKG2D) is constitutively expressed on mouse NK

287   cells, NKT cells and some other cells (Abel et al., 2018; Al Dulaimi et al., 2018), but does not

288   get expressed on naïve mouse CD8 T cells (Prajapati et al., 2018). Human CD8 T cells always

289   express NKG2D on their surface, but mouse CD8 T cells only express it upon activation

290   (Wensveen *et al.*, 2018) NKG2D is activated by NKG2D ligands (Raulet et al., 2013), and

291   NKG2D ligands are relatively restricted to malignant or transformed cells (Raulet, 2003; Raulet

292   *et al.*, 2013). In order to determine whether loss of *TCF-7* affects NKG2D expression and anti-

293   tumor responses, we analyzed NKG2D expression in CD8 T cells. We measured NKG2D

294   expression by flow cytometry before and at different time points after CD3/CD28 activation

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295   (Karimi et al., 2015). We found that CD8 T cells from *TCF-7* cKO mice had significantly

296   increased expression of NKG2D on the cells surface compared to CD8 T cells from WT mice,

297   before stimulation **(Fig.3A)**. Next, we wanted to examine whether NKG2D expression was

298   further upregulated on CD8 T cells from *TCF-7* cKO mice compared to CD8 T cells from WT

299   mice after stimulation. CD8 T cells were cultured with 2.5ug/ml anti-CD3 and 2.5ug/ml anti-

300   CD28 antibodies for 24, 48, or 72 hours. These cultured cells were examined for NKG2D

301   expression by flow cytometry. We observed an increase in NKG2D expression on CD8 T cells

302   from both WT and *TCF-7* cKO mice in a time-dependent manner, and at all time points,

303   expression of NKG2D was higher for cells from *TCF-7* cKO mice **(Fig.3B).** There was no

304   difference in the viability of the cells or CD8 T cell numbers before or after the culture

305   **(Supp.Fig.2A-B).**

306   We also wanted to compare the Granzyme B expression in CD8+, NKG2D+ T cells from

307   *TCF-7* cKO and WT mice (Chu et al., 2013). We did not observe any Granzyme B expression in

308   CD8 T cells before stimulation **(Fig.3C).** Only 24 hours after stimulation, we observed

309   Granzyme B expression in T cells from both strains, peaking at 48 hours post-stimulation with

310   no difference between strains of mice **(Fig.3C)**. After 72 hours post-stimulation, CD8 T cells

311   from WT mice had significantly reduced Granzyme B expression compared to *TCF-7* cKO CD8

312   T cells **(Fig.3C)**. We also confirmed total Granzyme B expression in CD8 T cells from *TCF-7*

313   cKO mice, in the presence and absence of CD3/CD28 stimulation, using Western blotting. Total

314   Granzyme B expression was upregulated in CD8 T cells from *TCF-7* cKO mice compared to WT

315   mice **(Fig.3D-E).** These data demonstrated that CD8 T cells from *TCF-7* cKO mice may

316   maintain anti-tumor responses by killing the target cells with an NKG2D-mediated mechanism,

317   and by persistent upregulation of Granzyme B expression (Liu et al., 2022; Wang et al., 2022).

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318   Next, we wanted to examine the functional consequences of upregulation of NKG2D

319   expression on CD8 T cells from *TCF-7* cKO mice. We utilized an in *vitro* cytotoxicity assay,

320   where we used anti-NKG2D neutralizing antibody. We isolated CD8 T cells from WT and *TCF-*

321   *7* cKO mice and cultured them for 48 hours with anti-CD3/anti-CD28 antibodies in order to

322   induce optimal NKG2D expression in CD8 T cells. CD8 T cells from *TCF-7* cKO and WT mice

323   were then cultured with tumor target A20 cells (Edinger *et al.*, 2003b) in a 40:1 ratio of tumor

324   cells to CD8 T cells, along with anti-NKG2D antibody or isotype control antibody for 4 hours.

325   We used the A20 cell line as a tumor target because it is known for expressing NKG2D ligands

326   including Rae1, H60, and MULT1(Karimi *et al.*, 2015; Nishimura et al., 2008). Triplicate wells

327   were averaged and percent lysis was calculated from the data using the following equation: %

328   specific lysis = 100 × (spontaneous death bioluminescence – test bioluminescence)/ (spontaneous

329   death bioluminescence – maximal killing bioluminescence) (Karimi et al., 2014).

330   Our data showed that the addition of anti-NKG2D antibody significantly reduced the

331   cytotoxicity of CD8 T cells from *TCF-7* cKO mice, whereas addition of isotype control had no

332   effect on cytotoxicity of the CD8 T cells from *TCF-7* cKO mice **(Fig.3F).** In contrast, the

333   addition of anti-NKG2D antibody (Karimi *et al.*, 2015) did not change cytotoxicity of the CD8 T

334   cells from WT mice **(Fig.3F).** These data further support the idea that *TCF-7* cKO CD8 T cells

335   maintain their anti-tumor activity through an NKG2D-mediated mechanism. Taking into account

336   that normal tissue does not express NKG2D ligands on the surface and that primarily malignant

337   and transformed cells upregulate these ligands, this could explain why CD8 T cells from *TCF-7*

338   cKO mice cause less GVHD but maintain their anti-tumor activity (Nishimura *et al.*, 2008).

339

340 **Loss of *TCF-7* alters cytokine production, chemokine expression, and expression of**

341 **exhaustion markers by mature CD8 T cells.** We confirmed that CD8 T cells from *TCF-7* cKO

342 mice mediate cytolytic function primarily through NKG2D. Next, we wanted to examine the

343 mechanism behind why CD8 T cells from *TCF-7* cKO mice induce less GVHD. One of the

344 hallmarks of GVHD is the release of pro-inflammatory cytokines by alloactivated donor T cells,

345 eventually leading to cytokine storm (Lynch Kelly et al., 2015; Mohty et al., 2005). We

346 examined whether loss of *TCF-7* in donor CD8 T cells led to changes in cytokine production,

347 thereby affecting GVHD damage. We allotransplanted lethally irradiated BALB/c mice as

348 described above. Recipient mice were transplanted with 1.5×10^6 WT or *TCF-7* cKO CD8 donor

349 T cells. Recipients were sacrificed at day 7 post-transplant. Splenocytes were isolated and

350 restimulated by 6 hours of culture with PBS (control) or anti-CD3/anti-CD28 (stimulation),

351 along with Golgiplug. Afterwards, the cultured cells were stained with antibodies against $H2K^b$,

352 CD3, CD4, CD8, TNF-α, and IFN-γ. Our data showed that production of TNF-α by donor CD8

353 T cells trended toward decreasing when *TCF-7* was lost **(Supp. Fig. 3A)**. In contrast, IFN-γ

354 trended toward increasing upon loss of *TCF-7* in CD8 T cells **(Supp. Fig. 3B)**.

355 　　　　We also obtained serum from cardiac blood of recipient mice at day 7 post-transplant and

356 tested it with a mouse Th cytokine ELISA panel (LEGENDplex kit from Biolegend) (Mammadli

357 *et al.*, 2020; Mammadli *et al.*, 2021d). Levels of TNF-α and IFN-γ in serum of recipient mice

358 given *TCF-7* cKO CD8 T cells were lower than in mice given WT CD8 T cells at day 7 **(Fig.**

359 **4A)**. In contrast, the serum levels of IL-2 in mice given *TCF-7* cKO CD8 T cells was higher than

360 in mice given WT CD8 T cells at day 7 **(Fig. 4A)**. At day 14 post-transplant, the reduction in

361 TNF-α and IFN-γ levels observed at day 7 for *TCF-7* cKO-transplanted mice was preserved **(Fig.**

362 **4B)**. We observed a trend towards decreased serum levels of IL-2 in mice given *TCF-7* cKO

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363 CD8 T cells compared with mice given WT CD8 T cells at day 14 post-transplant, opposite of

364 the effect observed on day 7 post-transplant **(Fig.4B)**. This suggests that *TCF-7* cKO CD8 T

365 cells may be capable of IFN-γ and TNF-α production at the same level as WT CD8 T cells when

366 restimulated, but in reality, produce less IFN-γ and TNF-α than WT cells when allotransplanted.

367   In order for GVHD to persist, donor T cells must proliferate in secondary lymphoid

368 organs and target organs (Beilhack et al., 2005; Ferrara, 2014). Naive and effector T cells drive

369 GVHD, but they are short-lived and must be replaced to maintain an alloresponse (Jiang et al.,

370 2021; Jiang et al., 2014). Also, given that memory cells are increased among CD8 T cells when

371 *TCF-7* is lost, we hypothesized that activation and/or exhaustion of these cells may also be

372 affected. Ki-67 (Blessin et al., 2021) is a marker of T cell activation and proliferation, and TOX

373 (Khan et al., 2019) and PD-1 are markers of exhaustion (Ahn et al., 2018). Therefore, we wanted

374 to determine the Ki-67, TOX, and PD-1 expression levels on WT and *TCF-7* cKO CD8 T cells *in*

375 *vitro*. We cultured splenocytes from either WT mice or *TCF-7* cKO mice *in vitro* with anti-CD3

376 and anti-CD28 antibodies for 24, 48, and 72 hours. We did not observe any difference in Ki-67

377 expression in cells that were not stimulated, but the CD8 T cells from *TCF-7* cKO mice that were

378 stimulated for 72 hours *in vitro* showed significant upregulation of Ki-67 expression (**Fig. 4 C-**

379 **D**), suggesting that CD8 T cells from *TCF-7* cKO mice could potentially proliferate more than

380 CD8 T cells from WT mice when restimulated**.** We also observed the same trend of increased

381 expression for PD-1 at 72 hours post-stimulation **(Fig.4E-F)**. There were no differences in

382 expression of TOX at any time points *in vitro* when CD8 T cells from *TCF-7* cKO mice were

383 compared to WT **(Supp.Fig.3C).**

384   Next, we checked the expression of these markers *in vivo* from donor cells that were allo-

385 transplanted in recipient Balb/c mice as described earlier. At day 7 post-transplant, splenocytes

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386   were isolated and Ki-67, TOX, and PD-1 expression were detected by flow cytometry. We

387   observed that donor CD8+T cells from *TCF-7* cKO mice expressed more TOX compared to WT,

388   suggesting that donor T cells *TCF-7* cKO mice were more exhausted following

389   allotransplantation **(Fig.5G-H)**. We did not observe any statistically significant differences in Ki-

390   67 or PD-1 expression at day 7 post-transplant **(Supp.Fig.3D-E).** Taken together these data

391   suggest that CD8 T cells from *TCF-7* cKO mice could be more exhausted than WT CD8 T cells

392   both *in vivo* and *in vitro*.

393   One of the major functions of alloactivated T cells is migration from spleen to

394   GVHD target organs, including liver and small intestine(Beilhack *et al.*, 2005; Ferrara, 2014).

395   Expression of chemokines and chemokine receptors is a critical aspect of T cell migration to

396   target organs. To determine whether expression of these molecules was affected by loss of *TCF-*

397   *7* in CD8 T cells, we FACS sorted pre- and post-transplanted donor CD8 T cells from WT or

398   *TCF-7* cKO mice (spleen only for pre-transplant, spleen, or liver for post-transplant). We then

399   extracted RNA from the cells, converted it to cDNA, and performed qPCR using a 96-well

400   mouse chemokine/chemokine receptor plate (Thermo Fisher). As expected, expression of these

401   markers was generally upregulated in alloactivated cells. Expression of these markers was

402   generally higher in *TCF-7* cKO CD8 T cells from pre-transplant spleen compared to WT pre-

403   transplant spleen **(Supp. Fig. 4A)**, but these markers were generally downregulated in *TCF-7*

404   cKO CD8 T cells from post-transplant liver and spleen compared to WT cells **(Supp. Fig. 4B-**

405   **C)**. Therefore, *TCF-7* controls expression of CD8 T cell chemokine/chemokine receptors.

406

407   **Loss of *TCF-7* in donor CD8 T cells led to decreased damage to the GVHD target organs.**

408   During GVHD, host tissues are damaged by the activity of alloactivated T cells. To determine

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409   whether damage to target organs of GVHD (skin, liver, and small intestine) was altered by loss

410   of *TCF-7* in donor T cells, we collected organs from mice allotransplanted as described above

411   (Beilhack *et al.*, 2005; Mammadli *et al.*, 2021a; Mammadli *et al.*, 2021d; Weeks *et al.*, 2021). At

412   day 7 and day 21 post-transplant, we collected pieces of skin, small intestine, and liver from

413   recipient BALB/c mice. These organs were fixed, sectioned, stained with hematoxylin and eosin

414   (H&E), and analyzed by a pathologist (L.S.) **(Fig. 5)**. At day 7, *TCF-7* cKO mice showed

415   significantly less inflammatory infiltrates in all the organs. We observed much less inflammatory

416   infiltrates in the bile duct epithelium of the portal triad (black arrows showing the interlobular

417   bile ducts) in the liver of the *TCF-7* cKO-transplanted recipients compared with WT-transplanted

418   recipients **(Fig. 5A)**. In the small intestines, no apoptotic bodies were seen in the crypts of the

419   small intestine in the *TCF-7* cKO CD8 T cell-transplanted mice, while frequent apoptotic bodies

420   were present in the WT CD8 T cell-transplanted mice at day 7 post-transplantation (black

421   arrows) **(Fig. 5B)**. In the skin, a mild increase in inflammatory cells was observed in the dermis

422   of the WT CD8 T transplanted mice, while the dermis of the *TCF-7* cKO CD8 T transplanted

423   mice appears normal at day 7 post-transplantation **(Fig. 5C)**.

424   Again, at day 21 post-transplant, *TCF-7* cKO CD8 T cell transplanted mice showed

425   significant less inflammatory infiltrates in all the sectioned GVHD target organs. We observed

426   much less inflammatory infiltrates involving the bile duct epithelium of the portal triad (black

427   arrows showing the interlobular bile ducts) in the liver of the *TCF-7* cKO CD8 T cell-

428   transplanted mice compared with WT CD8 T cell-transplanted mice **(Fig. 5D)**. At day 21 post-

429   transplant, no apoptotic bodies were seen in the crypts of the small intestines of the *TCF-7* cKO

430   CD8 T cell transplanted mice, while few apoptotic bodies are present in the small intestines of

431   the WT CD8 T cell transplanted mice (black arrows) **(Fig. 5E)**. A marked increase in

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432 inflammatory cells (red circle) and destruction of the adnexal glands was observed in the dermis

433 of the WT CD8 T cell-transplanted recipients, while the dermis of the *TCF-7* cKO CD8 T cell

434 transplanted recipients showed only a mild increase in dermal inflammatory cells, with

435 preservation of adnexal glands **(Fig. 5F)**. Altogether, these data suggest that *TCF-7* normally

436 promotes GVHD damage to healthy tissues and is indispensable for T cell-driven damage. Thus,

437 loss of *TCF-7* in donor T cells leads to reduced severity and persistence of GVHD over time.

438

439 ***TCF-7* alters the transcriptomic signature of alloactivated T cells.** Given that the phenotype

440 and functions of donor T cells, as well as disease outcomes, were significantly altered by loss of

441 *TCF-7* on donor cells, we sought to determine what specific gene changes occurred to support

442 this. We allotransplanted recipient BALB/c mice with $1X10^6$ donor CD3 T cells as above, and

443 FACS-sorted pre- and post-transplant WT or *TCF-7* cKO donor CD8 T cells, which were stored

444 in Trizol and transcriptionally profiled. When we analyzed the genetic profile of the pre-

445 transplanted CD8 T cells from *TCF-7* cKO and WT mice, we were not able to determine any

446 differentially expressed genes (DEGs, FDR<0.1). However, when we performed Gene Set

447 Enrichment analysis (GSEA) using the Hallmark pathways collection from Molecular Signatures

448 Database (MSigDB), we identified that cytokine signaling pathways like TNF-a Signaling via

449 NF-κβ and Interferon gamma response were enriched in pre-transplanted CD8 T cells from WT

450 mice compared with cells from *TCF-7* cKO mice **(Supp.Fig.5A-C)**. Meanwhile, a number of

451 pathways involved in cell cycle also were enriched in WT cells versus *TCF-7* cKO pre-

452 transplanted CD8 T cells, like the P53 pathway, G2M checkpoint, DNA repair, and Myc targets

453 pathways **(Supp.Fig.5A).** MTOR signaling, Allograft rejection, and Oxidative phosphorylation

454 pathways were also enriched in pre-transplant CD8 T cells from WT mice **(Supp.Fig.5A,6D-F),**

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455   suggesting that loss of *TCF-7* alters the transcriptional profile of CD8 T cell towards decreased

456   cytokine release while also altering the cell cycle, leading to a lessening of the alloactivation

457   responses.

458   When we analyzed the post-transplanted CD8 T cells which were alloactivated *in vivo*, we

459   identified 2548 differentially expressed genes (DEGs; FDR<0.1) when comparing *TCF-7* cKO

460   cells to WT cells **(Fig. 6A-B).** A majority of the DEGs (2000 genes) were downregulated

461   (module 2 in heatmap) and only 548 genes (module 1 in heatmap) were upregulated in post-

462   transplant CD8 T cells from *TCF-7* cKO mice compared to WT mice **(Fig. 6A-B).** We analyzed

463   both up- and downregulated DEGs for the Gene Ontology (GO) enrichment analysis using

464   Functional Annotation Chart tools, selecting only the top 20 GO BP (Biological Process) terms

465   in the Database for Visualization and Integrative Discovery (DAVID)(Huang da et al., 2009;

466   Sherman et al., 2022). The analysis showed that the differentially expressed genes in post-

467   transplant CD8 T cells from *TCF-7* cKO mice compared to WT were involved in Cell Cycle,

468   Cell-cell adhesion, Cell division, Apoptotic process, Antigen processing and presentation via

469   MHC class I, TCR signaling, Regulation of NF-κB signaling, Metabolic process, and others

470   **(Supp.Fig.6).** Once we knew which processes these DEGs played a role in, we pulled out the top

471   genes that were altered for each GO BP term that we were interested in. When we looked at the

472   top 35 DEGs based on P-value that were altered in Cell cycle, we observed that a majority of

473   them were downregulated in *TCF-7* cKO compared to WT **(Fig.6C).** We also looked at the top

474   30 genes based on P-value that were altered in Apoptotic processes **(Fig.6D)**, which also showed

475   that most of the genes were downregulated in CD8 T cells in *TCF-7* cKO mice. When we looked

476   at the top 30 genes that play a role in metabolic processes, we observed that only 1 gene was

477   upregulated, and the rest were downregulated in *in vivo* alloactivated CD8 T cells from *TCF-7*

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478   cKO mice **(Fig. 6E).** While the upregulated genes in the NF-κB pathway were Fasl, Ubd, Chuk

479   and others, the downregulated genes were Rela, Irf3, Traf2, Mavs, Tradd, Nod1, Tnfrsf1a, and

480   Trim25 **(Fig.6F)**.

481   Gene Set Enrichment analysis (GSEA) using the Hallmark pathways identified that

482   signaling pathways like PI3K-AKT-MTOR Signaling and TNFA Signaling via NF-κβ were

483   enriched in post-transplanted CD8 T cell from WT mice compared to cells from TCF-7 cKO

484   mice **(Supp.Fig.7A-B)**. GSEA analysis using the C2 canonical pathways showed that a number

485   of cytokines signaling pathways involving IL-1, IL-2, IL-4, and IL-7 were enriched in post-

486   transplanted CD8 T cells from WT mice compared to cells from *TCF-7* cKO mice **(Fig.6G)**.

487   While Cell cycle pathways were enriched in CD8 T cells from *TCF-7* cKO mice compared to

488   cells from WT mice, Apoptosis and Cell adhesion pathways were enriched in post-transplanted

489   CD8 T cells from WT mice **(Fig.6H).** Meanwhile, a number of cells signaling pathways were

490   also enriched in WT CD8 T cells compared to *TCF-7* cKO cells, such as TCR signaling, Toll

491   like receptor signaling, Jak-Stat signaling, ERK-MAPK signaling, and NF-kB pathways **(Fig.6I).**

492   We also analyzed the genes that were altered in KEGG pathways, which revealed that

493   DEGs that were altered in *in vivo* alloactivated CD8 T cells from *TCF-7* cKO mice (compared to

494   WT) were involved in pathways like Cell cycle, DNA replication, Metabolic pathways, Natural

495   killer mediated cytotoxicity, TCR signaling, JAK-STAT signaling, Chemokine receptor

496   signaling, and others **(Fig.7A).** Specifically, Klrk1 gene for NKG2D on Natural killer mediated

497   cytotoxicity pathway were enriched in allo-activated *TCF-7* deficient CD8+ T cells compared to

498   WT CD8+ T cells. Clustering of genes that were affected in TCR signaling showed that while

499   AKT1, AKT2, Pik3r5, Zap70, LCK, Lat, PLCγ1, Pdcd1, Vav1, Rela, Mapk3, Nfkbia, and Nfatc1

500   were downregulated, Ifng and Ptprc were upregulated in post-transplanted CD8 T cells from

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501   *TCF-7* cKO mice **(Fig.7B).** The JAK/STAT signaling pathway is important for cytokine

502   production and for the response of T cells to cytokines. Analysis revealed that IL2RB, JAK3,

503   STAT5B, STAT3, STAT1, Cish, Il2rb, Socs3, and Socs1 genes were downregulated in the JAK-

504   STAT pathway for *TCF-7* cKO CD8 T cells compared to WT **(Fig.7C)**.

505   In order to confirm these changes in genes, we isolated CD8 T cells from WT and *TCF-7*

506   *cKO* mice and determined the baseline level protein expression of LCK, ZAP70, LAT, ITK,

507   PLCγ1, ERK1-2, Jak2, Jak3, Stat3, p65-rela, AKT, and actin in unstimulated and 10 minute-anti-

508   CD3/CD28-stimulated samples. Data from non-stimulated samples revealed that protein

509   expression of most of the markers was downregulated in CD8 T cells from *TCF-7* cKO mice

510   compared to WT mice; only ZAP70, JAK3 and AKT were not affected by loss of *TCF-7*

511   **(Fig.7D, Supp.Fig.8A).** We observed even more robust differences in samples that were

512   stimulated with anti-CD3/CD28 for 10 minutes, and again only ZAP70 and AKT were

513   unaffected by loss of *TCF-7* **(Fig.7E, Supp.Fig.8B).** Altogether, the data from RNA sequencing

514   analysis and Western blots of stimulated and unstimulated samples showed attenuation of TCR

515   signaling and many other pathways in CD8 T cells from *TCF-7* cKO mice. These results help to

516   explain why CD8 T cells from *TCF-7* cKO mice cannot induce GVHD as severely as CD8 T

517   cells from WT mice.

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Discussion

T Cell Factor-1 (*TCF-7*) is a critical regulatory transcription factor in T cell development and

functions (Zuniga-Pflucker, 2004). *TCF-7* is known to be important for T cell development, as

well as activation in some contexts (Yu et al., 2010). *TCF-7* has been extensively studied in viral

infection(Escobar *et al.*, 2020; He *et al.*, 2016; Im *et al.*, 2016; Kurtulus *et al.*, 2019; Miller *et al.*,

2019; Siddiqui *et al.*, 2019; Utzschneider *et al.*, 2016; Weber *et al.*, 2011; Wu *et al.*, 2016).

However, the role of (*TCF-7*) in a mouse model of allogeneic transplant has not been

investigated. Using a murine allogeneic transplant model, we have shown that CD8 T cells from

TCF-7 cKO effectively clear tumor cells without inducing GVHD by producing significantly less

inflammatory cytokines (Ju *et al.*, 2005; Seif *et al.*, 2017). Our data also uncovered that CD8 T

cells from *TCF-7* cKO mice cause significantly less tissue damage in GVHD target organs

(Bleakley *et al.*, 2012; Breems and Lowenberg, 2005). Here, we show that loss of *TCF-7* alters a

number of CD8 T cell functions, and while it is dispensable for anti-tumor responses, it is

essential for host tissue damage, cytokine production and signaling, and gene expression, playing

a role in a number of immunological and biological pathways during alloactivation. (Yu *et al.*,

2010). This murine model allows us to study T cell function, clinical outcomes, and gene

expression all in one model. Here, we showed that *TCF-7*-deficiency alters the phenotype of

CD8 T cells by upregulating CD44 and CD122. We and other have shown that innate memory-

like CD8 T cells expressing CD12hi and CD44 hi, Eomes and T-bet ameliorate GVHD

development (Huang et al., 2019; Karimi *et al.*, 2014; Mammadli *et al.*, 2020; Mammadli *et al.*,

2021d; Zheng et al., 2009). The role of central (CD44^{hi}, CD62L^{hi}) and effector (CD44^{hi},

CD62L^{low}) memory phenotypes has been investigated previously (Huang *et al.*, 2019; Zheng *et

al.*, 2009). Our data demonstrated that *TCF-7* significantly impacts CD8 T cell central memory

phenotypes, suggesting that *TCF-7* might be a regulator for T cell activation. While effector and

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546   naive cells are known to cause severe GVHD, central memory cells are often associated with less

547   severe disease (Dutt *et al.*, 2011; Tugues et al., 2018; Zheng *et al.*, 2009). Our findings

548   suggesting that this phenotypic change in *TCF-7 cKO* cells may be beneficial for reducing

549   disease severity. Our experiments in mixed bone marrow chimeras showed that bone marrow-

550   derived CD8 T cells from WT and or *TCF-7* cKO mice developed in the same thymus have a

551   similar phenotype to each other, and a different phenotype than CD8 T cells from naïve WT or

552   *TCF-7* cKO mice. We found that the upregulation of activation marker expression like Eomes,

553   CD122, and the effector memory phenotype is primarily cell-intrinsic, with changes to other

554   markers being either cell-extrinsic or primarily cell-intrinsic with other extrinsic effects.

555   Donor T cells are crucial for target organ injury in graft-versus-host disease (GVHD).

556   These alloactivated T cells proliferate, migrate to target organs (liver, skin, and small intestine),

557   and produce cytokines during GVHD(Bastien et al., 2012; Reddy and Ferrara, 2008; Villarroel et

558   al., 2014). Our data showed that tissue damage in liver, skin, and small intestine (all target

559   organs) was reduced by loss of *TCF-7* in donor CD8 T cells at all timepoints. This shows that

560   *TCF-7* in donor T cells is required for GVHD damage and persistence over time.

561   Donor T cells eliminate tumor cells (GVL) but also cause graft-versus-host disease

562   (GVHD) (Bleakley *et al.*, 2012; Tugues *et al.*, 2018). Our data showed that CD8 T cells from

563   *TCF-7* cKO mice were able to clear tumor without causing GVHD, suggesting that *TCF-7* is

564   dispensable for anti-tumor responses. Our data revealed that CD8 T cells from *TCF-7* cKO mice

565   mediate cytolytic function via NKG2D. We also confirmed this hypothesis by blocking the

566   surface NKG2D by anti-NKG2D antibody on CD8 T cells from *TCF-7* cKO and WT mice and

567   performing an *in vitro* cytotoxicity assay. While the anti-tumor response of TCF1-deficient CD8

568   T cell against A20 cells (which express the NKG2D ligands like Rae1, H60, and MULT1)

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569 (Nishimura *et al.*, 2008) was diminished, cytotoxicity of WT CD8 T cells was not affected.

570 Upregulation of the *KLRK1* gene (also known as NKG2D) in alloactivated *TCF-7* deficient

571 CD8+ donor T cells further confirmed our hypothesis. Furthermore, the increase in Granzyme B

572 expression in *TCF-7* cKO CD8 T cells by flow cytometry and Western blot also provides

573 evidence as to why the anti-tumor response is preserved despite weakened TCR signaling in

574 TCF-7 cKO CD8 T cells (Presotto et al., 2017).

575 Once we had a clear mechanism for the GVL effect, we looked at functions that were

576 altered in CD8 T cells from *TCF-7* cKO mice that could produce the attenuated GVHD effect.

577 We discovered that the serum levels of cytokines like TNFα and IFNγ were decreased in *TCF-7*

578 deficient CD8 T cell-transplanted mice. Published data has shown the CD8 T cell lacking *TCF-7*

579 develop exhaustion while clearing viral infection(Gautam et al., 2019; Seo et al., 2019) While the

580 expression of TOX, an exhaustion marker, was not affected in freshly isolated or *in vitro*-

581 stimulated *TCF-7* deficient CD8 T cells, alloactivated *TCF-7*-deficient CD8 donor T cells

582 upregulated TOX at day 7 post-transplant (Scott et al., 2019). Another exhaustion marker, PD-1,

583 was upregulated after 72 hours in *in vitro*-stimulated CD8 T cells from TCF-7 cKO mice, but *in*

584 *vivo* expression of PD-1 in donor T cells was not increased (Wang et al., 2019a; Xu et al., 2019).

585 This could be explained by differences between alloactivation *in vivo* and TCR-mediated

586 activation *in vitro.* Ki-67, a proliferation marker, was also altered by loss of *TCF-7*, suggesting

587 that CD8 T cells from *TCF-7* cKO mice may proliferate more compared to WT CD8 T cells

588 (Sobecki et al., 2016). We also confirmed that CD8 T cells from *TCF-7* cKO mice cause less

589 tissue damage to the target organs, and loss of *TCF-7* alters chemokine receptor expression both

590 pre- and post-transplant, which could explain why these cells cause less severe GVHD with

591 increased survival of recipient mice (Ferrara, 2014).

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592   These studies provide evidence for how *TCF-7* regulates the functions of peripheral T

593   cells. The phenotype caused by loss of *TCF-7* is clinically optimal, because it allows for

594   clearance of residual malignant cells while limiting the risk of life-threatening GVHD damage

595   (Guinan et al., 1999). This observation, coupled with the increase in exhaustion of *TCF-7* cKO

596   donor CD8 T cells, suggests that donor cells lacking *TCF-7* are highly activated and cytotoxic to

597   malignant cells early on following transplant, but quickly become exhausted, limiting GVHD

598   progression.

599   Finally, to identify the changes to the genetic program of donor T cells that occurred in the

600   absence of *TCF-7*, we performed RNA sequencing analysis on pre- and post-transplant donor T

601   cells. Even though we could not identify the Differentially Expressed Genes (DEGs) in pre-

602   transplanted T cells (which we attribute to technical problems), gene set enrichment analysis

603   revealed that loss of *TCF-7* alters the genetic signature of pre-transplanted CD8 T cells. A

604   number of signaling pathways involved in cytokine production and cell cycle were enriched in

605   pre-transplanted CD8 T cell from WT mice compared to cells from *TCF-7* cKO mice, suggesting

606   that loss of *TCF-7* alters the transcriptional profile of the CD8 T cell towards decreased cytokine

607   release, while altering the cell cycle in baseline and leading to the lessening of the allo-activation

608   response. Meanwhile, 2548 DEGs (FDR<0.1) were identified when comparing post-transplanted

609   CD8 T cells from *TCF-7* cKO mice and WT mice. Both GO Annotation analysis of DEGs and

610   Gene Set Enrichment analysis revealed that a number of pathways such as Cell cycle, DNA

611   replication, Metabolic pathways, TCR signaling, JAK-STAT signaling, and Chemokine receptor

612   signaling were enriched in post-transplanted CD8 T cells from WT mice compared with cells

613   from *TCF-7* cKO mice. Transcriptomic analysis also revealed that KLRK-1 gene for NKG2D

614   were upregulated in allo-activated *TCF-7* cKO CD8+ T cells compared to the WT CD8+T cells,

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615 which confirming our findings in in-vitro cytotoxicity assay and flow cytometry data. These

616 findings suggest that loss of *TCF-7* leads to changes in the transcriptomic profile of the CD8 T

617 cells towards producing less cytokines and attenuating the T cell response, while increasing

618 cytotoxicity. Chemokine receptor pathways were also enriched in alloactivated WT CD8 T cells

619 which confirmed the qPCR analysis of chemokine receptors. The decrease in chemokine

620 receptors helps to explain why we observed less tissue damage in GVHD target organs after allo-

621 transplantation, and attenuated GVHD persistence, in mice given *TCF-7* cKO CD8 T cells. By

622 using Western blotting, we also confirmed changes in TCR and JAK/STAT signaling before and

623 after stimulation with anti-CD3/CD28, which helps to explain why we saw less serum cytokines

624 at day 7 and day 14 post-transplant in mice given *TCF-7* cKO CD8 T cells. This also helps to

625 explain why GVHD didn't persist in recipients given CD8 T cells which lack *TCF-7*.

626   Altogether, these data suggest that *TCF-7* is a major transcription factor that plays a role

627 in T cell development. Our work shows that *TCF-7* is dispensable for cytotoxic function of

628 mature alloactivated CD8 T cells but is indispensable for GVHD. TCR, JAK-STAT, and NF-

629 κB signaling as well as cytokine production, these findings will help to establish an

630 understanding of *TCF-7* as a critical factor in the GVHD/GVL regulatory network of CD8 T

631 cells.

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Materials and Methods

Mouse Models. For transplant, the following female donor mice were used: B6-Ly5.1

(CD45.1+, "WT" or B6.SJL-Ptprca Pepcb/BoyCrl, 494 from Charles River), C57Bl/6J

(CD45.2+, "WT", 000664 from Jackson Laboratories), or Tcf7 flox x CD4cre (referred to here as

" *TCF-7 cKO*" (Ma *et al.*, 2012) , obtained from Dr. Jyoti Misra Sen at NIH by permission of Dr.

Howard Xue, and bred in-house),. These donor mice were age-matched to each other and to

recipients as closely as possible. BALB/c female mice (CR:028 from Charles River, age 6-8

weeks or older) were used as recipient mice for transplant experiments, and Thy1.1 mice

(B6.PL-Thy1a/CyJ, 000406 from Jackson Labs) were used as recipient mice for chimera

experiments.

Allotransplant and Tumor Models. BALB/c recipient mice were irradiated with two doses of

400 cGy of x-rays (total dose 800 cGy) and rested for at least 12 hours between doses. Mice

were also rested for 4 hours prior to transplantation. T cells (total CD3+ or CD8+) were

separated from WT and *TCF-7 cKO* spleens using CD90.2 or CD8 microbeads and LS columns

(Miltenyi, CD8: 130-117-044, CD90.2: 130-121-278, LS: 130-042-401). $1X10^6$ donor cells

(unless otherwise mentioned) were injected IV into the tail vein in PBS, along with $10X10^6$ WT

bone marrow cells. Bone marrow was T-cell depleted with CD90.2 MACS beads (130-121-278

from Miltenyi) and LD columns (130-042-901 from Miltenyi). For short-term experiments, at

day 7 post-transplant, recipient mice were euthanized and serum, spleen, lymph nodes, small

intestine, or liver were collected, depending on the experiment. For GVHD and GVL

experiments, recipient mice were also given $2X10^6$ luciferase expressing B-cell lymphoma (A-

20) (Edinger *et al.*, 2003a). Recipient mice were weighed, given a clinical score, and imaged

28

665   using the IVIS 50 imaging system three times per week until day 70 or longer. Clinical scores

666   were composed of scores for skin integrity, fur texture, posture, activity, diarrhea, and weight

667   loss. Imaging was done by injecting recipients I.P. with D-luciferin to detect tumor cell

668   bioluminescence. To produce mixed bone marrow chimeras, Thy1.1 mice were lethally

669   irradiated and reconstituted with a 1:4 (WT: *TCF-7 cKO*) mixture of bone marrow cells (total

670   50×10^6 cells), then rested for 9 weeks. At 9 weeks, tail vein blood was collected and checked by

671   flow cytometry for CD45.1 and CD45.2 to ensure reconstitution with both donor cell types. At

672   10 weeks, mice were used for phenotyping experiments.

673

674   **Flow Cytometry, Sorting, and Phenotyping.** Splenocytes (or cells from other organs) were

675   obtained from WT or *TCF-7* cKO mice or recipient allotransplanted mice. Lymphocytes were

676   obtained and lysed with RBC Lysis Buffer (00-4333-57 from eBioscience) to remove red blood

677   cells if needed. Cells were then stained with extracellular markers for 30 min on ice in MACS

678   buffer (1X PBS with EDTA and 4g/L BSA). If intracellular markers were used, the cells were

679   then fixed and permeabilized using the Fix/Perm Concentrate and Fixation Diluent from FOXP3

680   Transcription Factor Staining Buffer Set (eBioscience cat. No. 00-5523-00). The cells were then

681   run on a BD LSR Fortessa cytometer and data were analyzed using FlowJo software v9

682   (Treestar). All antibodies were used at a 1:100 dilution. For FACS sorting, the same methods

683   were applied, and cells were run on a BD FACS Aria IIIu with cold-sorting blocks. Cells were

684   sorted into sorting media (10% FBS in RPMI) or Trizol, depending on the experiment.

685   Depending on the experiment, antibodies used were: anti-CD4 (FITC, PE, BV785, BV21), anti-

686   CD8 (FITC, PE, APC, PerCP, Pacific Blue, PE/Cy7), anti-CD3 (BV605 or APC/Cy7), anti-

687   H2Kb-Pacific Blue, anti-H2Kd-PE/Cy7, anti-CD122 (FITC or APC), anti-CD44 (APC or Pacific

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688   Blue), anti-CD62L (APC/Cy7), anti-TNF-α-FITC, anti-IFN-γ-APC, anti-Eomes (AF488 or

689   PE/Cy7), anti-T-bet-BV421, anti-CD45.2-PE/Dazzle594, anti-CD45.1-APC, anti-Ki67 (PE or

690   BV421), anti-PD1-BV785, anti-CTLA4-PE, NKG2D-BV711, Granzyme B-PE/Cy7.

691

692   **Histology.** Recipient mice were allotransplanted as described, and organs were removed for

693   histology at day 7, and day 21 post-transplant. Spleen, liver, small intestine, and skin (from ear

694   and back) were fixed, sectioned, and stained with H&E at Cornell University (

695   https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/anatomic-

696   pathology/services). A pathologist (L.S) analyzed the sections for T cell-induced damage.

697

698   **Cytokine Restimulation.** Recipient BALB/c mice were allotransplanted with 1.5×10^6 CD3

699   donor T cells and euthanized at day 7. Splenocytes were taken and cultured for 6 hours with

700   GolgiPlug (1:1000) and PBS (control) or anti-CD3 (1ug/mL)/anti-CD28 (2ug/mL) (TCR

701   stimulation) at 37 C and 7% CO2. After 6 hours of culture, the cells were stained for CD3, CD4,

702   CD8, H2K^b, TNF-α, and IFN-γ using the BD Cytokine Staining kit (BD Biosciences, 555028),

703   and run on a flow cytometer.

704

705   **LegendPLEX Serum ELISA Assay.** Serum from cardiac blood was collected from recipient

706   mice in the cytokine restimulation experiment. Serum was analyzed using the Biolegend

707   LEGENDPlex Assay Mouse Th Cytokine Panel kit (741043). This kit quantifies serum

708   concentrations of: IL-2 (T cell proliferation), IFN-γ and TNF-α (Th1 cells, inflammatory), IL-4,

709   IL-5, and IL-13 (Th2 cells), IL-10 (Treg cells, suppressive), IL-17A/F (Th17 cells), IL-21 (Tfh

710   cells), IL-22 (Th22 cells), IL-6 (acute/chronic inflammation/T cell survival factor), and IL-9

711   (Th2, Th17, iTreg, Th9 – skin/allergic/intestinal inflammation).

712

713   **Western blot.** Splenocytes from WT or *TCF-7* cKO donor mice were collected. CD8 T cells

714   were separated using CD8 MACS beads. CD8 T cells were either stimulated with 2.5ug/ml anti-

715   CD3 (Biolegend #100202) and anti-CD28 antibodies (Biolegend #102115) for 10 minutes or left

716   unstimulated. These cells were counted and lysed with RIPA Buffer (89900 from Thermo Fisher)

717   plus protease inhibitors (11697498001 from Millipore Sigma) and phosphatase inhibitors

718   (P5726-1ML and P0044-1ML from Millipore Sigma). The lysates were run on a Western blot

719   and probed for Perforin (Cell Signaling Technology #3693), Granzyme B (Cell Signaling

720   Technology #4275), LCK (Thermo Fisher PA5-34653), ZAP70(Cell Signaling Technology

721   #3165), LAT(Cell Signaling Technology # 45533), ITK (Thermo Fisher PA5-49363), PLCγ1

722   (Cell Signaling Technology #2822), ERK1-2 (Cell Signaling Technology #9107), JAK 2(Cell

723   Signaling Technology # 3230), JAK3 (Cell Signaling Technology #8863), STAT3 (Cell

724   Signaling Technology #9139), p65-Rela (Cell Signaling Technology #4764), AKT (Cell

725   Signaling Technology #9272), , and β-actin (Cell Signaling Technology #4970). All the western

726   blots repeated at least three times and one representative of each protein and quantification is

727   shown.

728

729   **qPCR Analysis.** To perform qPCR, BALB/c mice were allotransplanted as described ($1X10^6$

730   CD3 donor T cells). Pre-transplant donor cells and post-transplant (day 7) spleen and liver cells

731   from recipients were FACS-sorted to obtain CD8 donor cells. The cells were sorted into Trizol,

732   RNA was extracted using chloroform (https://www.nationwidechildrens.org/

31

733   Document/Get/93327), and eluted using the Qiagen RNEasy Mini kit (74104 from Qiagen).

734   Concentration was checked with a spectrophotometer, then RNA was converted to cDNA with

735   an Invitrogen Super Script IV First Strand synthesis System kit (18091050 from Invitrogen).

736   Final cDNA concentration was checked with a spectrophotometer, then cDNA was mixed with

737   Taqman Fast Advanced Master Mix (4444557 from Invitrogen) at a 10ng/μL cDNA

738   concentration. This master mix was added to premade 96 well TaqMan Array plates with

739   chemokine/chemokine receptor primers (Thermo Fisher, Mouse Chemokines & Receptors Array

740   plate, 4391524). qPCR was performed in a Quant Studio 3 thermocycler, and data were analyzed

741   using the Design and Analysis software v2.4 (provided by Thermo Fisher). Five separate

742   recipient mice were sorted, and cells were combined to make one sample for qPCR testing per

743   condition/organ.

744

745   **NKG2D expression and NKG2D mediated cytotoxicity in CD8 T cells.** To determine the

746   NKG2D expression in CD8 T cells, we obtained splenocytes from WT and *TCF-7* cKO mice and

747   stimulated T cells with 2.5ug/ml anti-CD3 (Biolegend #100202) and anti-CD28 antibodies

748   (Biolegend #102115) for 24, 48, or 72 hours in culture, or left them unstimulated. GolgiPlug

749   (1:1000) was added to stimulated samples for each time point, and samples were incubated at 37

750   C and 7% CO2. After 6 hours of culture, the cells were stained with LIVE/DEAD Aqua and for

751   CD3, CD8, NKG2D, and Granzyme B using the BD Cytokine Staining kit (BD Biosciences,

752   555028), and run on a flow cytometer. To assess the NKG2D mediated cytotoxicity, we used

753   luciferase-expressing A20 cells as target cells as described earlier. Effector cells (MACS-sorted

754   CD8 T cells from *TCF-7* cKO or WT mice) were incubated in 2.5μg/ml anti-CD3 and anti-CD28

755   coated plates for 48 hours to induce optimal NKG2D expression. Then effector cells were added

756   at 40:1 effector-to-target ratios and incubated at 37°C for 4 hours with the A20 cells. Anti-

757   NKG2D antibody (10 μg/mL, Bio X Cell #BE0334) or rat IgG1 isotype control antibody (10

758   μg/mL, Bio X Cell #BE0334) was added and incubated for 30 minutes before washing and

759   plating. Triplicate wells were averaged and percent lysis was calculated from the data using the

760   following equation: % specific lysis = 100 × (spontaneous death bioluminescence – test

761   bioluminescence)/(spontaneous death bioluminescence – maximal killing bioluminescence).

762

763   **Exhaustion/Activation Assay.** To determine the *in vitro* exhaustion and activation of CD8 T

764   cells, we obtained splenocytes from WT and *TCF-7* cKO mice and either activated them with

765   2.5ug/ml anti-CD3 (Biolegend #100202) and anti-CD28 antibodies (Biolegend #102115) for 24,

766   48, or 72 hours in culture, or left them unstimulated, and stained for CD3, CD8, Ki-67, Tox, and

767   PD-1 markers. To assess exhaustion and activation of *in vivo* donor CD8 T cells, recipient mice

768   were allotransplanted as before (1X10^6 CD3 donor T cells) and euthanized at day 7.

769   Lymphocytes were obtained from spleen, and stained for CD3, CD4, CD8, H2K^b, TOX, Ki-67

770   and PD-1 markers.

771

772   **DNA Extraction and PCR.** Donor mice were genotyped using PCR on DNA extracted from ear

773   punches. At 4 weeks of age mice were ear punched, and DNA was extracted using the Accustart

774   II Mouse Genotyping kit (95135-500 from Quanta Biosciences). Standard PCR reaction

775   conditions and primer sequences from Jackson Laboratories were used for CD4cre. For Tcf7,

776   primer sequences and reaction conditions were obtained from Dr. Jyoti Misra Sen of NIH.

777

33

778   **RNA Sequencing.** Recipient mice were allotransplanted as before ($1X10^6$ CD3 donor T cells),

779   except that donor CD8 T cells were also FACS-sorted prior to transplant. A sample of sorted

780   donor cells was also saved for pre-transplanted RNA sequencing in Trizol. At day 7 post-

781   transplant, donor CD8 T cells were FACS-sorted back from recipient spleen of *TCF-7* cKO and

782   WT transplanted mice. The cells were all sorted into Trizol, then RNA was extracted and

783   prepped by the Molecular Analysis Core (SUNY Upstate,

784   https://www.upstate.edu/research/facilities/molecular-analysis.php). Paired end sequencing was

785   done with an Illumina NovaSeq 6000 system at the University at Buffalo Genomics Core

786   (http://ubnextgencore.buffalo.edu). For data analysis we used the statistical computing

787   environment R (v4.0.4), the Bioconductor suite of packages for R, and R studio (v1.4.1106). We

788   calculated the transcript abundance by performing pseudoalignment using the Kallisto (Bray et

789   al., 2016) (version 0.46.2). Calculated Transcript per million (TPM) values were normalized and

790   fitted to a linear model by empirical Bayes method with the Voom (Law et al., 2014) and Limma

791   (Ritchie et al., 2015) R packages to determine Differentially expressed genes – DEGs (FDR<0.1,

792   after controlling for multiple testing using the Benjamini-Hochberg method). DEG's were used

793   for hierarchical clustering and heatmap generation in R. Gene Ontology enrichment analysis was

794   conducted using either the Function Annotation Chart tools using only GO BP and KEGG terms

795   in the Database for Visualization and Integrative Discovery (Huang da et al., 2009a; b) (Huang

796   da *et al.*, 2009a) DAVID enrichment scores >1.3 are equivalent to a P value<0.05. For Gene set

797   enrichment analysis (GSEA) we used Hallmark and C2 gene set collections of Molecular

798   Signatures Database (MsigDB) and cluster Profiler package in R. Data will be deposited on the

799   Gene Expression Omnibus (GEO) database for public access

800   https://www.ncbi.nlm.nih.gov/geo/

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801   The RNAseq experiment described here was performed as part of the experiment described in

802   other recent publications from our laboratory (Mammadli et al., 2021a; Mammadli et al., 2021b;

803   Mammadli et al., 2020; Mammadli et al., 2021c). Therefore, the data generated for WT pre- and

804   post-transplanted samples (CD4 and CD8) are the same as that shown in the papers mentioned,

805   but here, these data are compared to data for *Cat-Tg* mice(Mammadli *et al.*, 2021a).

806

807   **Statistical Analysis.** Unless otherwise noted in the figure legends, all numerical data are

808   presented as means and standard deviations with or without individual points. Analysis was done

809   in GraphPad Prism v7 or v9. Most data were analyzed with Student's t-test, one-way ANOVA,

810   or two-way ANOVA, with Tukey's multiple comparisons test for ANOVA methods, depending

811   on the number of groups. Kaplan-Meier survival analyses were done for survival experiments.

812   All tests were two-sided, and p-values less than or equal to ($\leq$) 0.05 were considered significant.

813   Transplant experiments used 3-5 mice per group, with at least two repeats. *Ex vivo* experiments

814   were done two to three times unless otherwise noted with at least three replicates per condition

815   each time. RNA seq was done once with three replicates per group and condition. qPCR was

816   done once with one sample per condition, and 5 mice were combined to make the one sample.

817   Western blots were done 3 times for unstimulated and 10 min anti-CD3/CD28 stimulated

818   samples, one experiment each is shown.

819

820   **Study Approval.** All animal studies were reviewed and approved by the IACUC at SUNY

821   Upstate Medical University. All procedures and experiments were performed according to these

822   approved protocols.

823

Author Contributions

824

825 RH, MM, and MK designed and conducted experiments, analyzed data, and wrote the

826 manuscript. MK assisted with scientific/technical research design. MK, and JMS edited the

827 manuscript. MK, SH assisted with data collection. LS performed histology analyses. QY

828 provided technical and scientific advice and assisted with data analysis.

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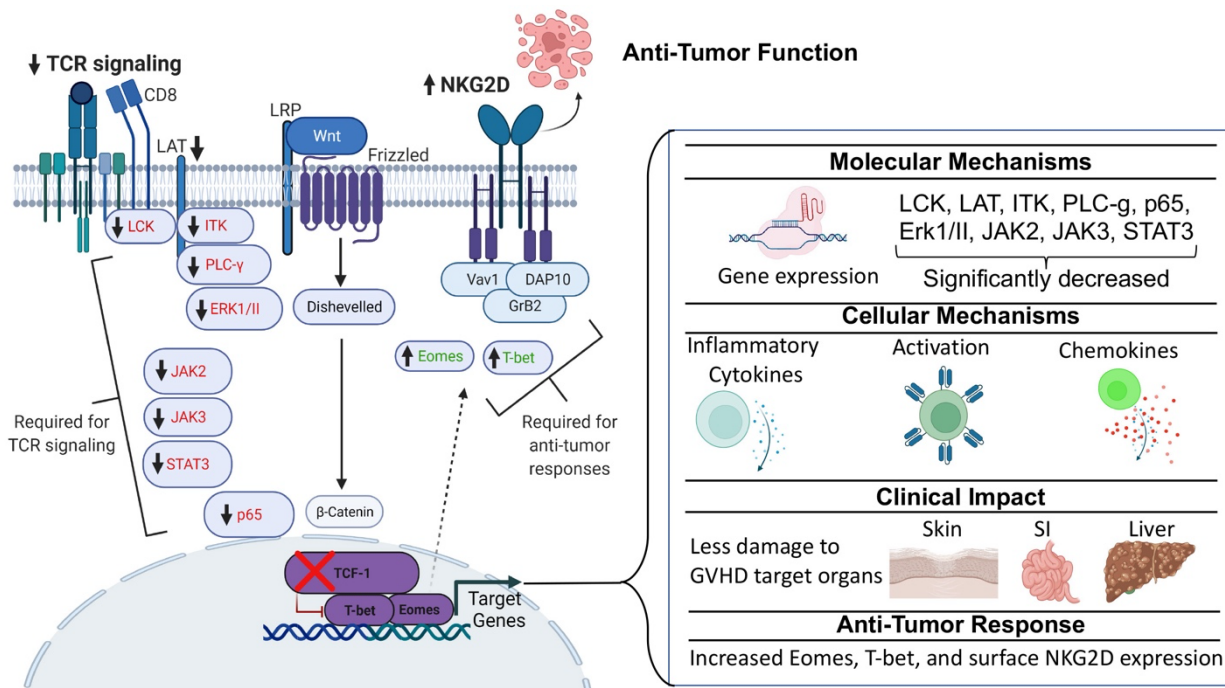
Summary Figure: *TCF-7* is required for persistent function of CD8 T cells but dispensable

for anti-tumor response. Here we have utilized a novel mouse model that lacks *TCF-7*

specifically on CD8 T cells for an allogeneic transplant model. We uncovered a molecular

mechanism of how *TCF-7* regulates key signaling pathways at both transcriptomic and protein

levels. These key molecules included LCK, LAT, ITK, PLC-γ1, p65, ERK I/II, and JAK/STAT

signaling. Next, we showed that the lack of *TCF-7* impacted phenotype, proinflammatory

cytokine production, chemokine expression, T cell activation. We provided clinical evidence for

how these changes impact GVHD target organs (skin, small intestine, and liver). Finally, we

provided evidence that *TCF-7* regulates NKG2D expression on mouse naïve and activated CD8

T cells. We have shown that CD8 T cells from *TCF-7* cKO mice mediate cytolytic functions via

NKG2D.

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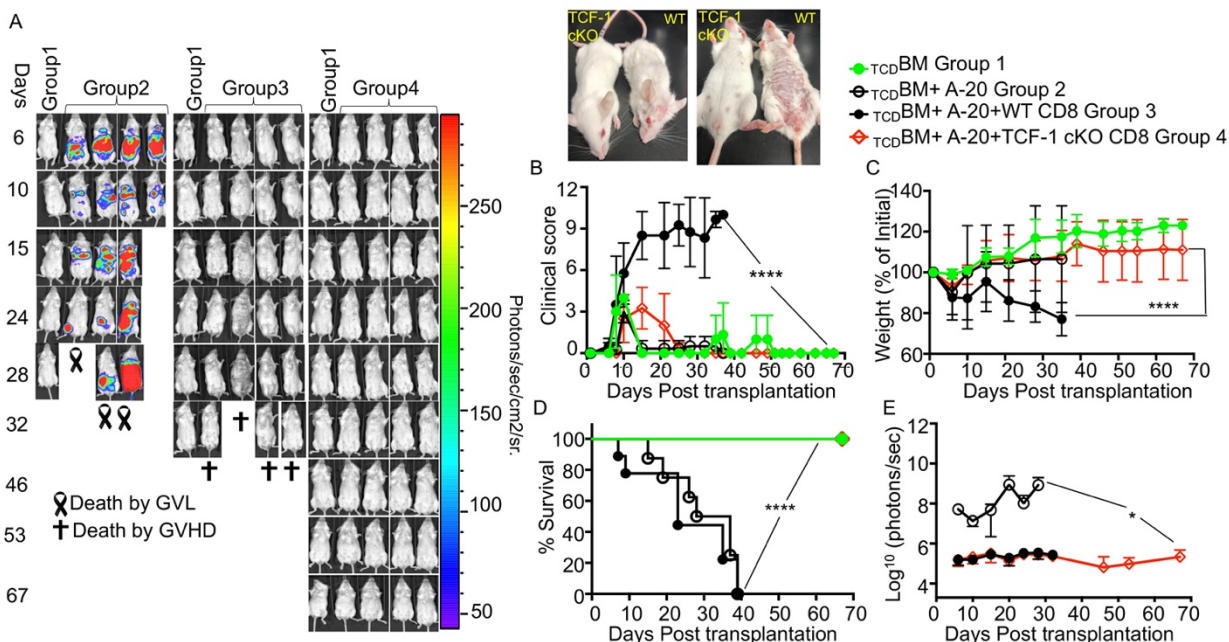
Figure 1: Loss of *TCF-7* in donor CD8 T cells reduces severity and persistence of GVHD symptoms. BALB/c recipient mice (MHC haplotype d) were lethally irradiated and allotransplanted with 1×10^6 CD8 T cells from WT or *TCF-7* cKO donor mice (MHC haplotype b), as well as 10×10^6 T cell-depleted bone marrow cells (BM) from WT mice (MHC haplotype b). Recipient mice were also given 2×10^5 luciferase-expressing A20 tumor cells **(A)** The recipient mice were imaged 1 time a week using IVIS50 for 70 days. Gross pictures of representatives of recipients transplanted with CD8 T cells from *TCF-7* cKO and WT mice at day 25 post-transplant are shown. **(B)** Recipient mice were also given a GVHD clinical score three times per week until day 70, based on combined scores of fur texture, activity level, weight loss, posture, skin integrity, and diarrhea. Mean and SD are plotted, analyzed by one-way ANOVA. **(C)** Weight changes of the recipient mice also were tracked over the time course of disease. **(D)** Survival for each group of recipient mice up to 70 days post-transplant, analyzed by Kaplan-Meier survival analysis. **(E)** Quantification of bioluminescence of tumor growth. For all graphs, * means p-value ≤ 0.05, ** means p-value ≤ 0.01, *** means p-value ≤ 0.001, and ****

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880 means p-value ≤ 0.0001 ($n = 3$ mice/group for BM alone: $n = 4$ experimental mice/group for all

881 other groups). Survival is a combination of two experiments. Note: Control mouse is one of the

882 mice from bone marrow only transplanted group used as a negative control for BLI.

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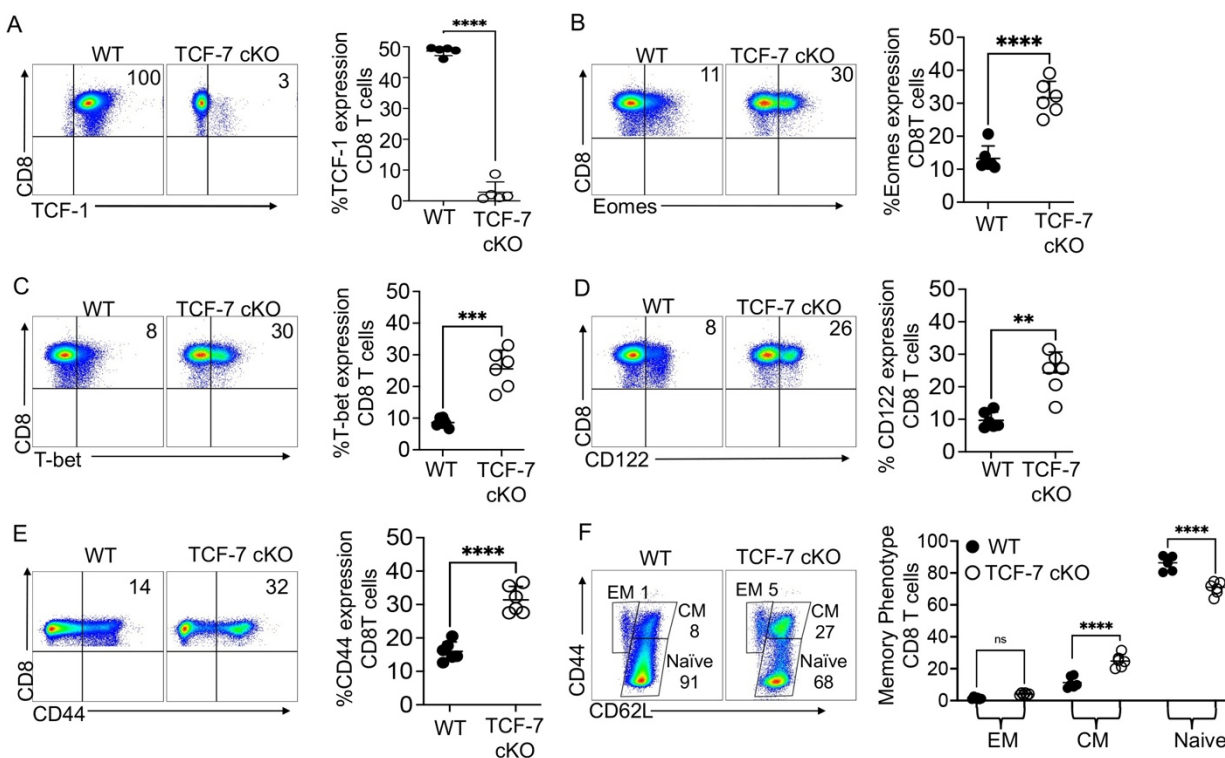
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885 **Figure 2. Loss of *TCF-7* changes the mature CD8 T cell phenotype.** Naive WT or *TCF-7*

886 cKO donor mice were euthanized and splenocytes were stained for flow cytometry phenotyping**.**

887 **(A)** Percent of CD8 T cells expressing *TCF-7* and quantified statistical analysis **(B)** Percent of

888 CD8 T cells expressing Eomes and quantified statistical analysis. **(C)** Percent of CD8 T cells

889 expressing T-bet and quantified statistical analysis. **(D)** Percent of CD8 T cells expressing

890 CD122 and quantified statistical analysis. **(E)** Percent of CD8 T cells expressing CD44 and

891 quantified statistical analysis. **(F)** Percent of CD8 T cells expressing central memory, effector

892 memory, or naive phenotypes and quantified statistical analysis. All data are plotted as individual

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893   points with mean and SD, all were analyzed with one-way ANOVA, or Student's t-test

894   (depending on groups). For all graphs, * means p-value ≤ 0.05, ** means p-value ≤ 0.01, ***

895   means p-value ≤ 0.001, and **** means p-value ≤ 0.0001. N=2-3 per group per experiment, with

896   combined data from 3 experiments shown.

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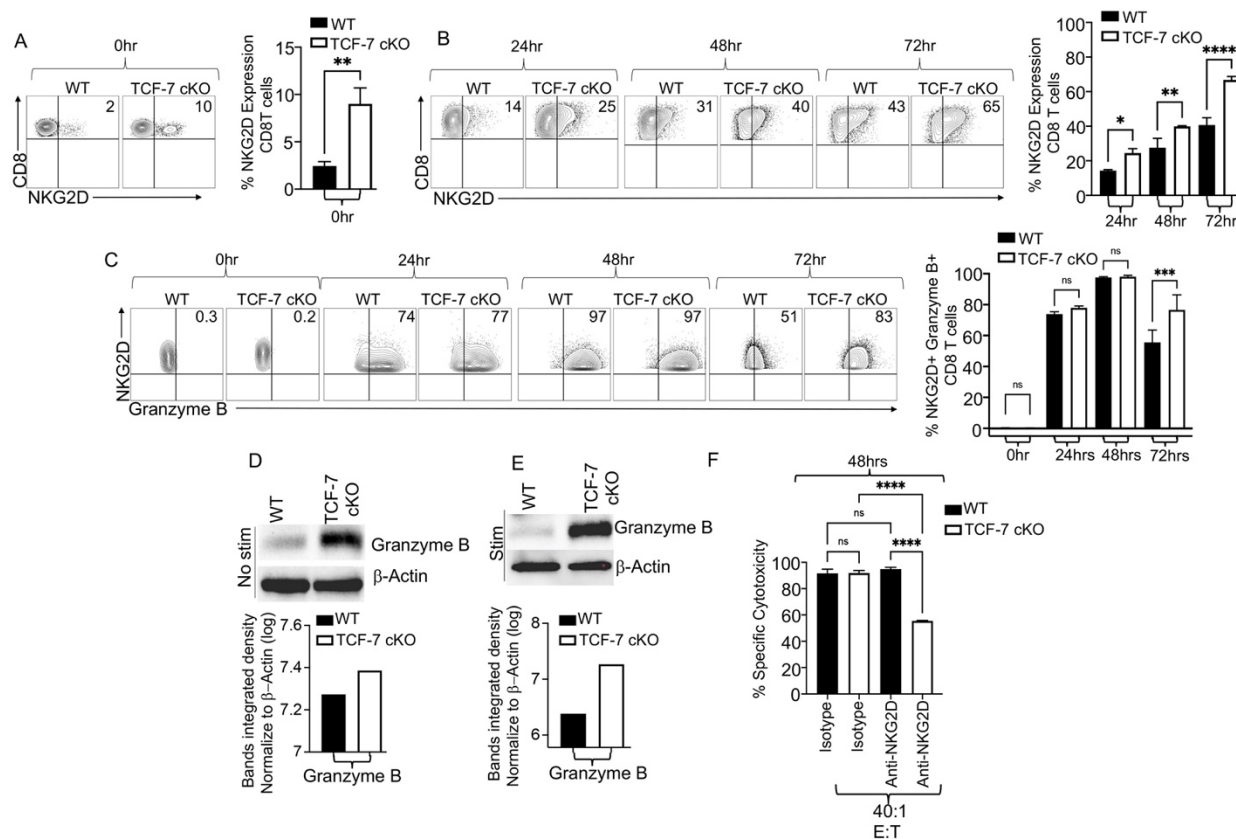
Figure 3: Loss of *TCF-7* alters cytotoxic mediator production by mature alloactivated CD8

899   **T cells.** Total splenocytes were isolated from *TCF-7* cKO or WT mice and either left

900   unstimulated or stimulated with anti-CD3/CD28 for 24, 48, or 72 hours in culture. GolgiPlug

901   (1:1000) was added to stimulated samples in each time point except 0-hour samples, and samples

902   were incubated at 37 C with 7% CO_2. After 6 hours of culture, the cells were stained with

903   LIVE/DEAD Aqua, and for CD3, CD8, NKG2D, and Granzyme B expression, as determined by

904   flow cytometry. **(A)** Percent of NKG2D expression in freshly isolated CD8 T cells from WT or

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905   *TCF-7* cKO mice and quantified statistical analysis. **(B)** Percent of NKG2D expression in 24, 48,

906   or 72 hour-stimulated CD8 T cells from WT and *TCF-7* cKO mice and quantified statistical

907   analysis. **(C)** Percent of Granzyme B expression in unstimulated or 24, 48, or 72 hour-stimulated

908   CD3+ CD8+ NKG2D+ T cells from WT and *TCF-7* cKO mice, and quantified statistical

909   analysis. **(D)** Granzyme B expression in unstimulated CD8 T cells from WT and *TCF-7* cKO

910   mice by Western blot, and bands' integrated density normalized to β-actin (quantified). **(E)**

911   Granzyme B expression in 10 minute-anti-CD3/CD28-stimulated CD8 T cells from WT and

912   *TCF-7* cKO mice by Western blot, and bands' integrated density normalized to β-actin

913   (quantified) **(F)** To assess the NKG2D-mediated cytotoxicity, we used luciferase-expressing A20

914   cells as target cells. Effector cells (MACS-sorted CD8 T cells from *TCF-7* cKO or WT mice)

915   were incubated in 2.5μg/ml anti-CD3 and anti-CD28 coated plates for 48 hours to induce optimal

916   NKG2D expression. Then effector cells were added at 40:1 effector-to-target ratios and

917   incubated at 37°C for 4 hours with A20 cells. Anti-NKG2D antibody (10 μg/mL) or rat IgG1

918   isotype control antibody (10 μg/mL) was added and incubated for 30 minutes before washing

919   and plating. Triplicate wells were averaged and percent lysis was calculated from the data using

920   the following equation: % specific lysis = 100 × (spontaneous death bioluminescence – test

921   bioluminescence)/ (spontaneous death bioluminescence – maximal killing

922   bioluminescence)(Karimi et al., 2014a; Karimi et al., 2015; Karimi et al., 2014b). N=4 per group

923   with one rappresentative of 2 experiments shown. Western blot were repeated twice and

924   representative examples are shown. All data are shown as individual points with mean and SD,

925   and were analyzed with Student's t-test or one-way ANOVA (depending on groups). * means p-

926   value ≤ 0.05, ** means p-value ≤ 0.01, and *** means p-value ≤ 0.001.

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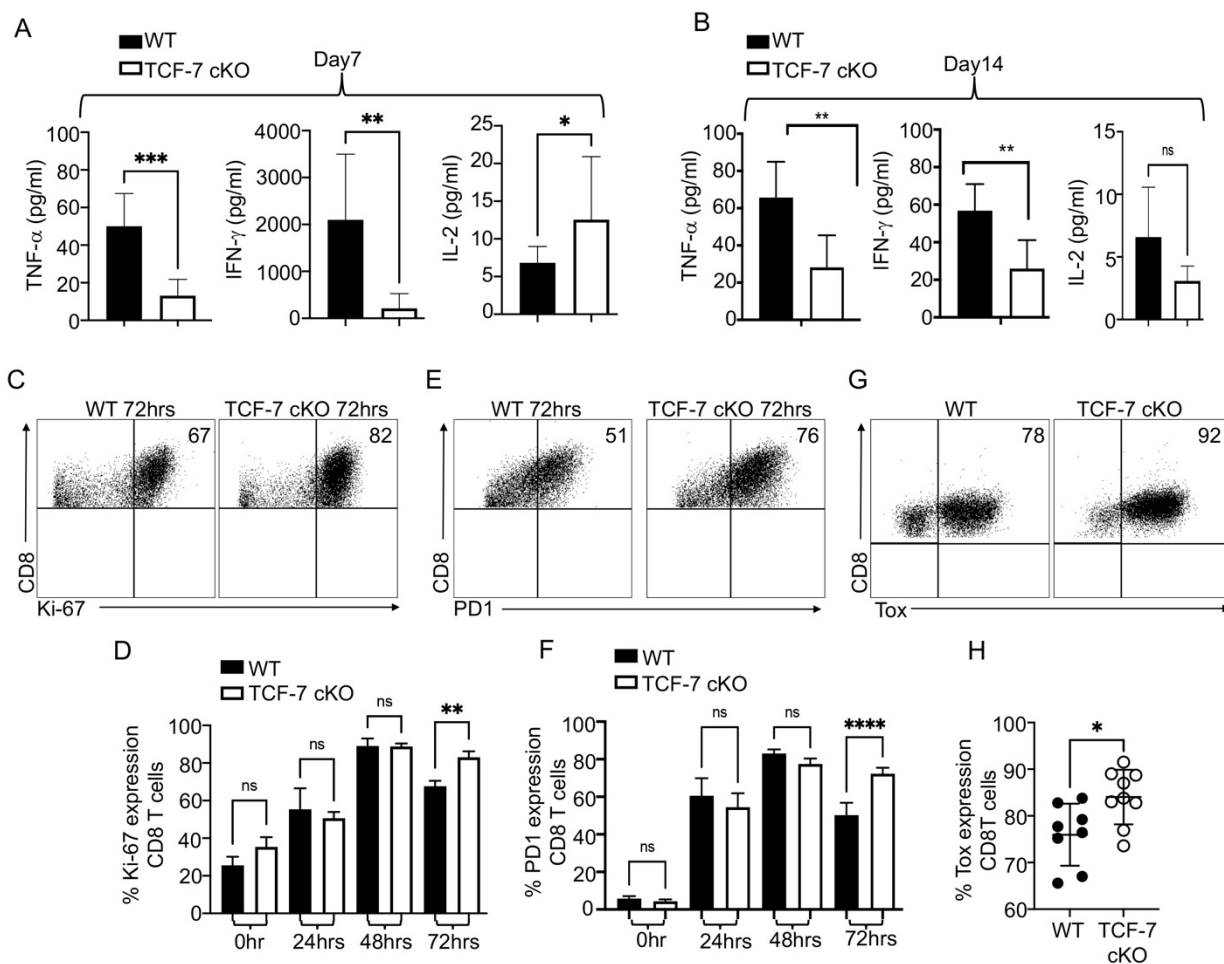
Fig.4. *TCF-7* controls cytokine production and exhaustion of mature alloactivated CD8 T cells. (A-B) Recipient mice were allotransplanted with 1.5×10^6 WT or *TCF-7* cKO donor CD3 T cells as before**.** At day 7 and day 14 post-transplant, serum was obtained from cardiac blood of allotransplanted recipient mice and tested for various cytokines using a LEGENDplex ELISA assay. **(A)** Serum levels (pg/mL) of TNF-α, IFN-γ, and IL-2 for WT and *TCF-7* cKO-transplanted mice at day 7 post-transplant. **(B)** Serum levels (pg/mL) of TNF-α, IFN-γ, and IL-2 for WT and *TCF-7* cKO-transplanted mice at day 14 post-transplant. **(C-F)** Splenocytes from *TCF-7* cKO and WT mice were obtained and stimulated with anti-CD3/CD28 antibodies for 24hrs, 48hrs, or 72hrs in culture and stained for Ki-67 and PD-1 or were stained immediately after isolation without stimulation. **(C)** Percent expression of Ki-67 in CD8 T cells after 72

42

938   hours of anti-CD3/CD28 stimulation in culture determined by flow cytometry. **(D)**

939   Quantification of the *in vitro* Ki-67 expression of CD8 T cells at different time points. **(E)**

940   Percent expression of PD-1 in CD8 T cells after 72 hours of anti-CD3/CD28 stimulation in

941   culture determined by flow cytometry. **(F)** Quantification of the *in vitro* PD-1 expression of CD8

942   T cells are different time points**. (G-H)** Balb/c mice were allotransplanted as before, with WT or

943   *TCF-7* cKO CD8 donor T cells. On day 7, spleens were removed from recipients, processed to

944   isolate lymphocytes, and spleen-derived lymphocytes from allotransplanted recipient mice were

945   stained for CD3, CD8, H2K^b, and TOX to identify exhausted T cells. **(G)** Percent expression of

946   TOX in CD8 T cells after allotransplantation (day 7 post-transplant) as determined by flow

947   cytometry. **(H)** Quantification of the TOX expression in *in vivo* donor CD8 T cells. N=3-5 per

948   group for **A-B** with two experiments shown. N=4 per group for **C-F,** one representative of two

949   experiments shown. N=3-5 per group with one representative experiment shown for **G-H**. All

950   data are shown as individual points with mean and SD, and were analyzed with Student's t-test

951   or two-way ANOVA (depending on groups). * means p-value ≤ 0.05, ** means p-value ≤ 0.01,

952   and *** means p-value ≤ 0.001.

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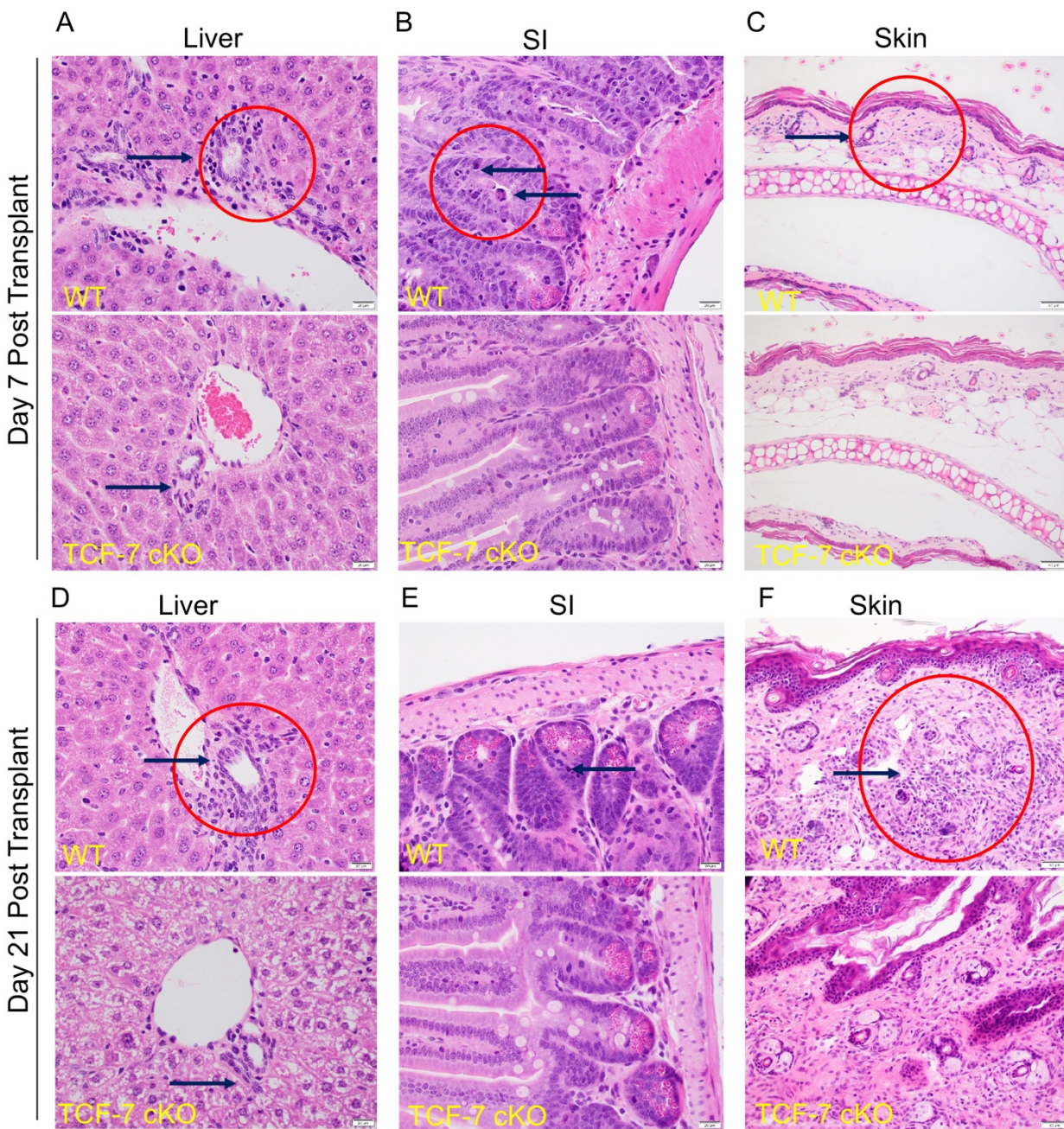
Fig.5. Loss of *TCF-7* in donor CD8 T cells decrease the damage to the GVHD target organs.

We collected organs from mice allotransplanted as described above. At day 7 and day 21 post-transplant, organs were taken from recipient mice for histology analyses. Skin, liver, spleen, and small intestine were sectioned, stained with H&E and analyzed by pathologist. Representative sections for each organ per group and timepoint are shown. **(A)** H&E staining of the liver of the

44

959 recipient mice at day 7 post-transplant (black arrows showing the interlobular bile ducts and red

960 circle showing inflammatory infiltrates). **(B)** H&E staining of the small intestines of the recipient

961 mice at day 7 post-transplant (black arrows showing the crypts of the small intestine and red

962 circle showing apoptotic bodies). **(C)** H&E staining of the skin of the recipient mice at day 7

963 post-transplant (black arrows showing the dermis of the skin and red circle showing increase of

964 inflammatory cells) **(D)** H&E staining of the liver of the recipient mice at day 21 post-transplant

965 (black arrows showing the interlobular bile ducts and red circle showing inflammatory

966 infiltrates). **(E)** H&E staining of the small intestines of the recipient mice at day 21 post-

967 transplant (blacks arrows showing apoptotic bodies). **(F)** H&E staining of the skin of the

968 recipient mice at day 21 (black arrows showing dermis of skin and red circle showing increased

969 inflammatory cells with destructed adnexal glands).
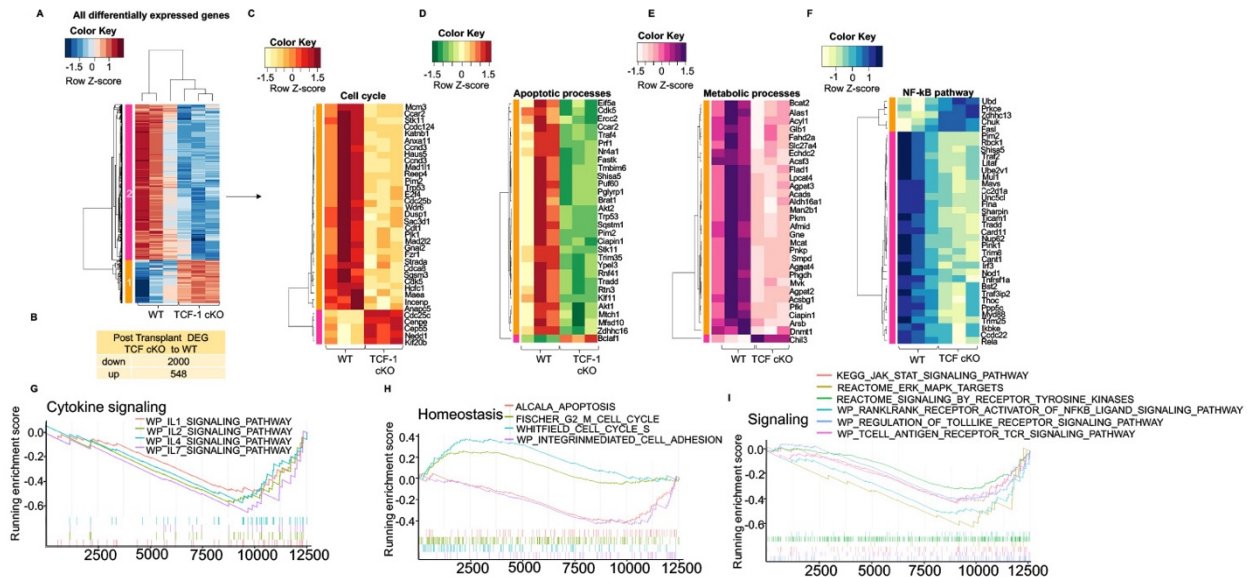
970

971 **Fig.6. Loss of *TCF-7* changes the genetic signature of alloactivated donor CD8 T cells.**

972 CD8 T cells from WT and *TCF-7* cKO mice were FACS sorted either into Trizol as pre-

973 transplanted samples, or into 10% FBS-containing media for transplantation into recipient mice

974 as described previously. At day 7 post-transplant, donor CD8 T cells were FACS-sorted back

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975   from recipient spleens of *TCF-7* cKO- and WT-transplanted mice. RNA was extracted and

976   prepped by the Molecular Analysis Core (SUNY Upstate). Paired end sequencing was done with

977   an Illumina NovaSeq 6000 system at the University at Buffalo Genomics Core. For data analysis,

978   we used the statistical computing environment R (v4.0.4), the Bioconductor suite of packages for

979   R, and RStudio (v1.4.1106). We calculated the transcript abundance by performing

980   pseudoalignment using Kallisto. **(A)** Hierarchical clustering of genes and samples, heatmap

981   illustrating the expression of the differentially expressing genes (DEG's; FDR<0.1) of post-

982   transplanted CD8 T cells *TCF-7* cKO compared to WT. **(B)** Table showing the number of up- or

983   downregulated DEGs in post-transplanted CD8 T cells (*TCF-7* cKO compared to WT). **(C)**

984   Heatmap showing top significant 35 differentially expressed genes that play a role in the Cell

985   Cycle pathway, identified using DAVID Functional Annotation Analysis and GO-BP terms in

986   post-transplanted CD8 T cells (*TCF-7* cKO compared to WT)**. (D)** Heatmap showing top

987   significant 30 differentially expressed genes that play a role in the Apoptotic Processes pathway,

988   identified using DAVID Functional Annotation Analysis and GO-BP terms in post-transplanted

989   CD8 T cells (*TCF-7* cKO compared to WT). **(E)** Heatmap showing top significant 30

990   differentially expressed genes that play a role in the Metabolic Processes pathway, identified

991   using DAVID Functional Annotation Analysis and GO-BP terms in post-transplanted CD8 T

992   cells (*TCF-7* cKO compared to WT). **(F)** Heatmap showing top significant 35 differentially

993   expressed genes that play a role in the NF-κB pathway, identified using DAVID Functional

994   Annotation Analysis and GO-BP terms in post-transplanted CD8 T cells (*TCF-7* cKO compared

995   to WT). **(G)** Gene set enrichment analysis (GSEA) enrichment plots of Cytokine signaling

996   pathways, including IL-1, IL-2, IL-4, and IL-7 signaling pathways from WP terms that are

997   enriched in post-transplanted CD8 T cells from WT mice (versus *TCF-7* cKO). Negative

46

998   Enrichment Score is an indicator of downregulation, and positive Enrichment Score is an

999   indicator of upregulation of the genes in the post-transplanted CD8 T cells from *TCF-7* cKo

1000   mice. DAVID enrichment scores >1.3 are equivalent to a P value<0.05 **(H)** GSEA enrichment

1001   plots of G2 to M Cell Cycle and Cell Cycle S pathways that are enriched in *TCF-7* cKO mice,

1002   and Integrin Mediated Cell Adhesion and Apoptosis pathways that are enriched in post-

1003   transplanted CD8 T cells from WT mice. **(I)** GSEA enrichment plots of JAK-STAT signaling,

1004   Toll like receptor signaling, T cell receptor signaling, ERK MAPK signaling, signaling by

1005   Tyrosine kinases, Rankl - Rank mediated NF-kB signaling pathways that are enriched in post-

1006   transplanted CD8 T cells from WT mice. Again, Negative Enrichment Score is an indicator of

1007   downregulation, and positive Enrichment Score is an indicator of upregulation of the genes in the

1008   post-transplanted CD8+ T cells from *TCF-7* cKo mice. DAVID enrichment scores >1.3 are

1009   equivalent to a P value<0.05.
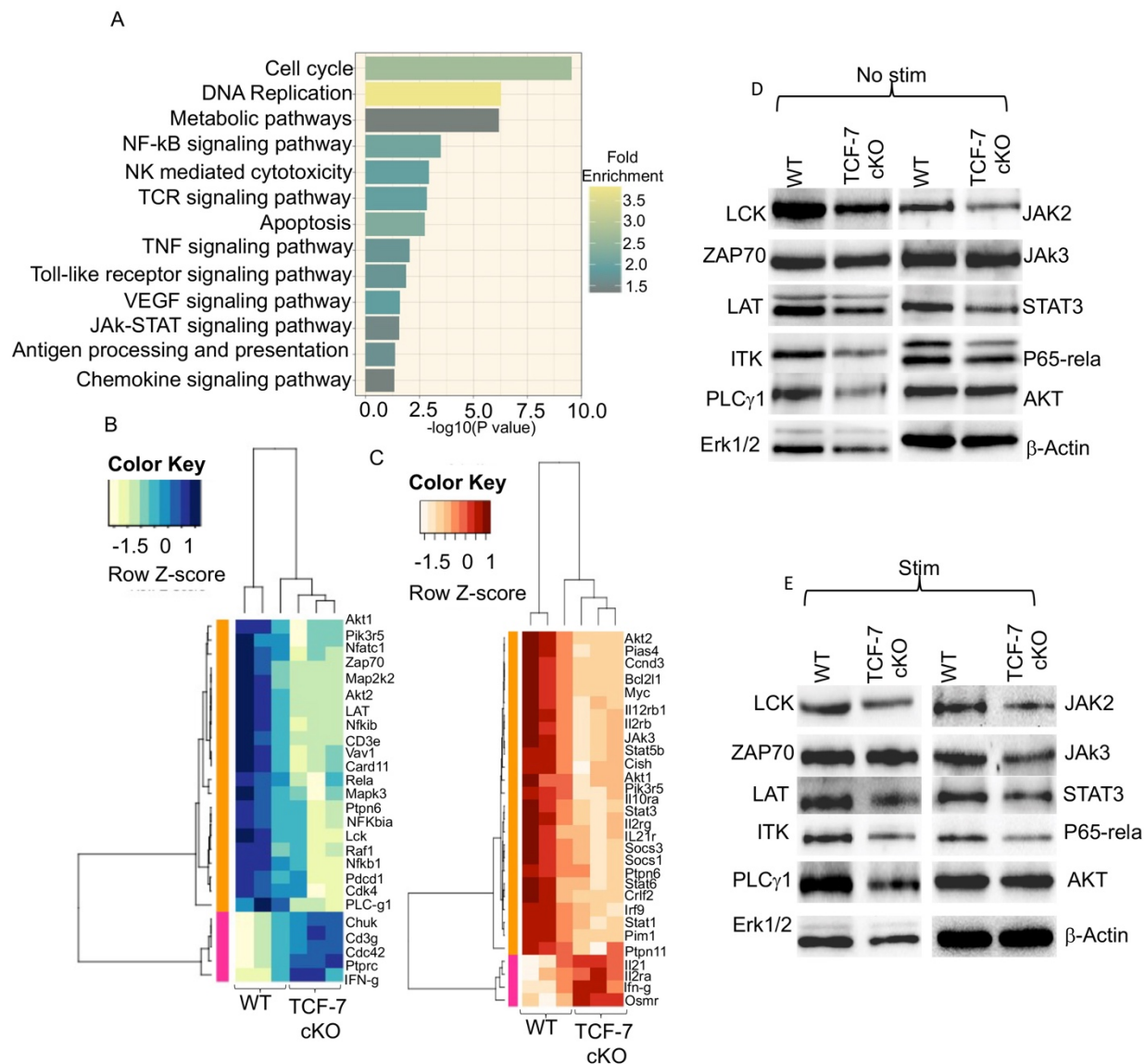
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Fig.7. Loss of *TCF-7* decrease TCR, JAK-STAT, and NF-kB signaling downstream. (A) A

bar plot showing KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways identified by

David functional annotation analysis. **(B)** Heatmap showing the altered genes in TCR signaling

pathway from KEGG pathways**. (C)** Heatmap showing the altered genes in JAK-STAT signaling

pathway from KEGG pathways. **(D)** Western blot showing the protein expression levels of LCK,

ZAP70, LAT, ITK, PLCγ1, ERK1/2, JAK2, JAK3, STAT3, P65-RelA, AKT, and β-actin of

freshly isolated CD8 T cell lysates from *TCF-7* cKO and WT mice**. (E)** Western blot showing

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1018   the protein expression levels of LCK, ZAP70, LAT, ITK, PLCγ1, ERK1/2, JAK2, JAK3,

1019   STAT3, P65-RelA, AKT, and β-actin in 10 minute-CD3/CD28-stimulated CD8 T cell lysates

1020   from *TCF-7* cKO and WT mice**.** All the western blots repeated at least three and one

1021   representative of each protein and quantification is shown.

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1024   **Supplemental Figure Legends:**
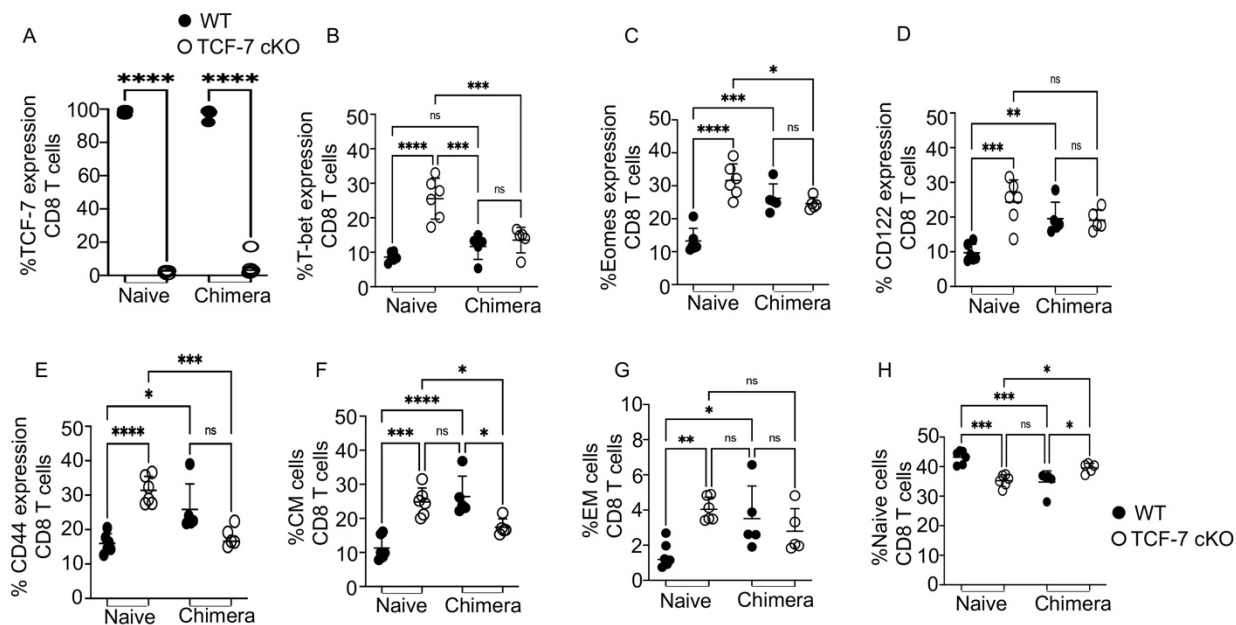
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1026   **Supp.Fig.1. Related to Fig2. Loss of *TCF-7* drives changes to mature CD8 T cell phenotype**

1027   **that are cell-intrinsic, with the possibility of extrinsic effects.** Bone marrow chimeras were

1028   developed by lethally irradiating Thy1.1 mice and reconstituting with a 1:4 (WT:*TCF-7* cKO)

1029   mixture of bone marrow cells. Blood was tested at 9 weeks to ensure reconstitution with both

1030   donor cell types, and splenocytes were used at 10 weeks for phenotyping by flow cytometry. **(A)**

1031   Percentage of CD8 T cells from chimeric and naive mice expressing *TCF-7*. **(B)** Percentage of

1032   CD8 T cells from chimeric and naive mice expressing T-bet. **(C)** Percentage of CD8 T cells from

1033   chimeric and naive mice expressing Eomes. **(D)** Percentage of CD8 T cells from chimeric and

1034   naive mice expressing CD122. **(E)** Percentage of CD8 T cells from chimeric and naive mice

1035   expressing CD44. **(F)** Percentage of CD8 T cells from chimeric and naive mice expressing

1036   central memory (CM) phenotype. **(G)** Percentage of CD8 T cells from chimeric and naive mice

1037   expressing effector memory (EM) phenotype. **(H)** Percentage of CD8 T cells from chimeric and

1038   naive mice expressing naïve phenotype. All data are plotted as individual points with mean and

50

1039   SD, all were analyzed with one-way ANOVA, or Student's t-test (depending on groups). For all

1040   graphs, * means p-value ≤ 0.05, *** means p-value ≤ 0.001, and **** means p-value ≤ 0.0001.

1041   For naïve cells 3 different experiments combined (N=2-3 per group of mice) and for chimera cell

1042   (N=5) with one experiment shown (done once).
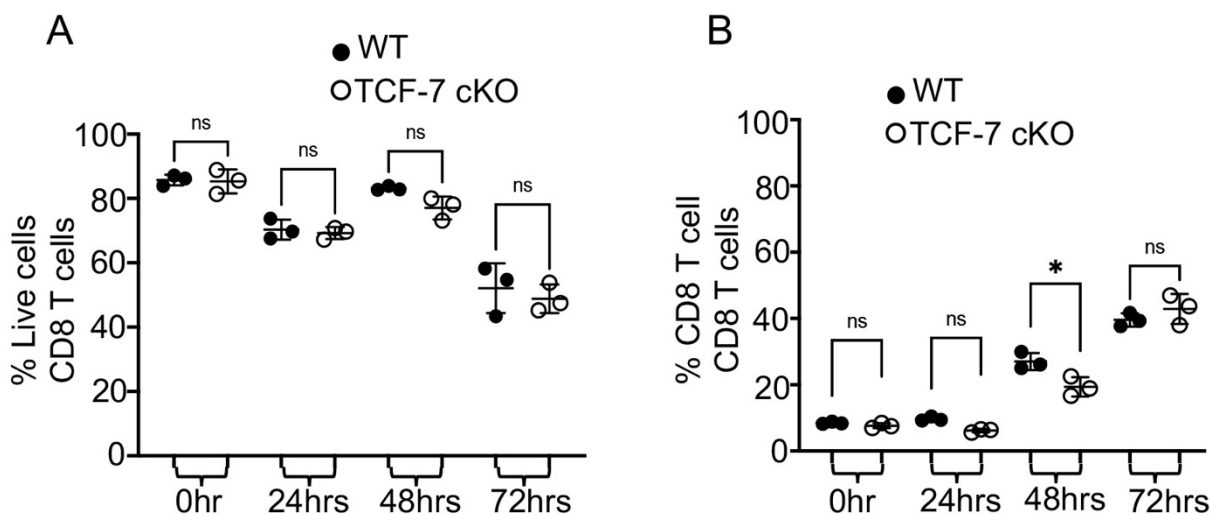
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Supp.Fig.2 Related to Fig.3. Cell viability and CD8 + T cell percent in NKG2D induction *in*

vitro. Total splenocytes were isolated from *TCF-7* cKO or WT mice and either left unstimulated

or stimulated with anti-CD3/CD28 for 24, 48, or 72 hrs in culture. GolgiPlug (1:1000) was added

to stimulated samples for each time point except 0 hr samples, and samples were incubated at 37

C with 7% CO_2. After 6 hours of culture, the cells were stained for CD3, CD8, NKG2D, and

Granzyme B expression, as determined by flow cytometry. **(A)** Quantification of percent of live

cells (dead cells that were positive for LIVE/DEAD Aqua excluded) for different time points of

stimulation. **(B)** Quantification of CD8 T cell percentages for different time points of

stimulation. N=4 per group with one representative of 2 experiments shown. All data are shown

as individual points with mean and SD, and were analyzed with two-way ANOVA (depending

on groups). * means p-value ≤ 0.05, ** means p-value ≤ 0.01, and *** means p-value ≤ 0.001.

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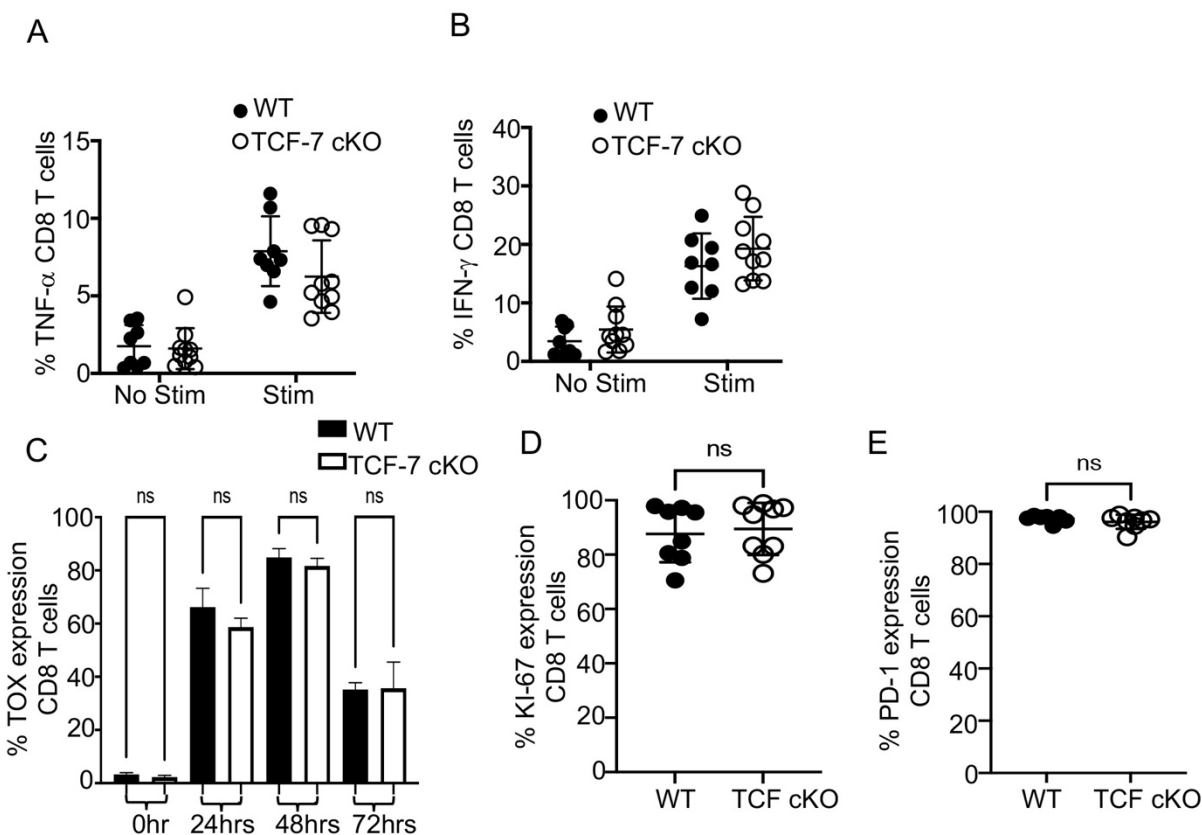
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Supp.Fig3. Related to Fig.5. *TCF-7* **controls cytokine production and exhaustion of mature**

alloactivated CD8 T cells. (A-B) Recipient mice were allotransplanted with $1.5X10^6$ WT or

TCF-7 cKO donor CD3 T cells as before. Splenocytes were taken at day 7 post-transplant,

restimulated by 6-hour culture with Golgi Plug and PBS (control) or anti-CD3/anti-CD28 (TCR

restim.), then stained for $H2K^b$, CD3, CD4, CD8, TNF-α, and IFN-γ. **(A)** TNF-α production and

(B) IFN-γ production by donor CD8 T cells, as measured by percent cytokine-positive donor

cells. **(C)** Splenocytes from *TCF-7* cKO and WT mice were obtained and either stimulated with

anti-CD3/CD28 antibodies for 24hrs, 48hrs, or 72hrs in culture and stained for TOX, or were

immediately stained after isolation without stimulation. Percent expression of TOX in CD8 T

cells after 72 hours of CD3/CD28 stimulation in culture, as determined by flow cytometry. **(D-E)**

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1067   Balb/c mice were allo-transplanted as before, with WT BM and WT or *TCF-7* cKO CD8 donor T

1068   cells. On day 7, spleens were removed from recipients, processed to isolate lymphocytes, and

1069   lymphocytes were stained for CD3, CD8, H2K^b, Ki-67, and PD-1 to identify proliferating and

1070   exhausted T cells, respectively. **(D)** Quantification of the Ki-67 expression in *in vivo* donor CD8

1071   T cells. (**E**) Quantification of the PD-1 expression in *in vivo* donor CD8 T cells. N=3-5 per group

1072   for **A-B and D-E** with two experiments shown. N=4 per group for **C,** one representative of two

1073   experiments shown. All data are shown as individual points with mean and SD, and were

1074   analyzed with Student's t-test or two-way ANOVA (depending on groups). * means p-value $\leq$

1075   0.05, ** means p-value $\leq$ 0.01, and *** means p-value $\leq$ 0.001.

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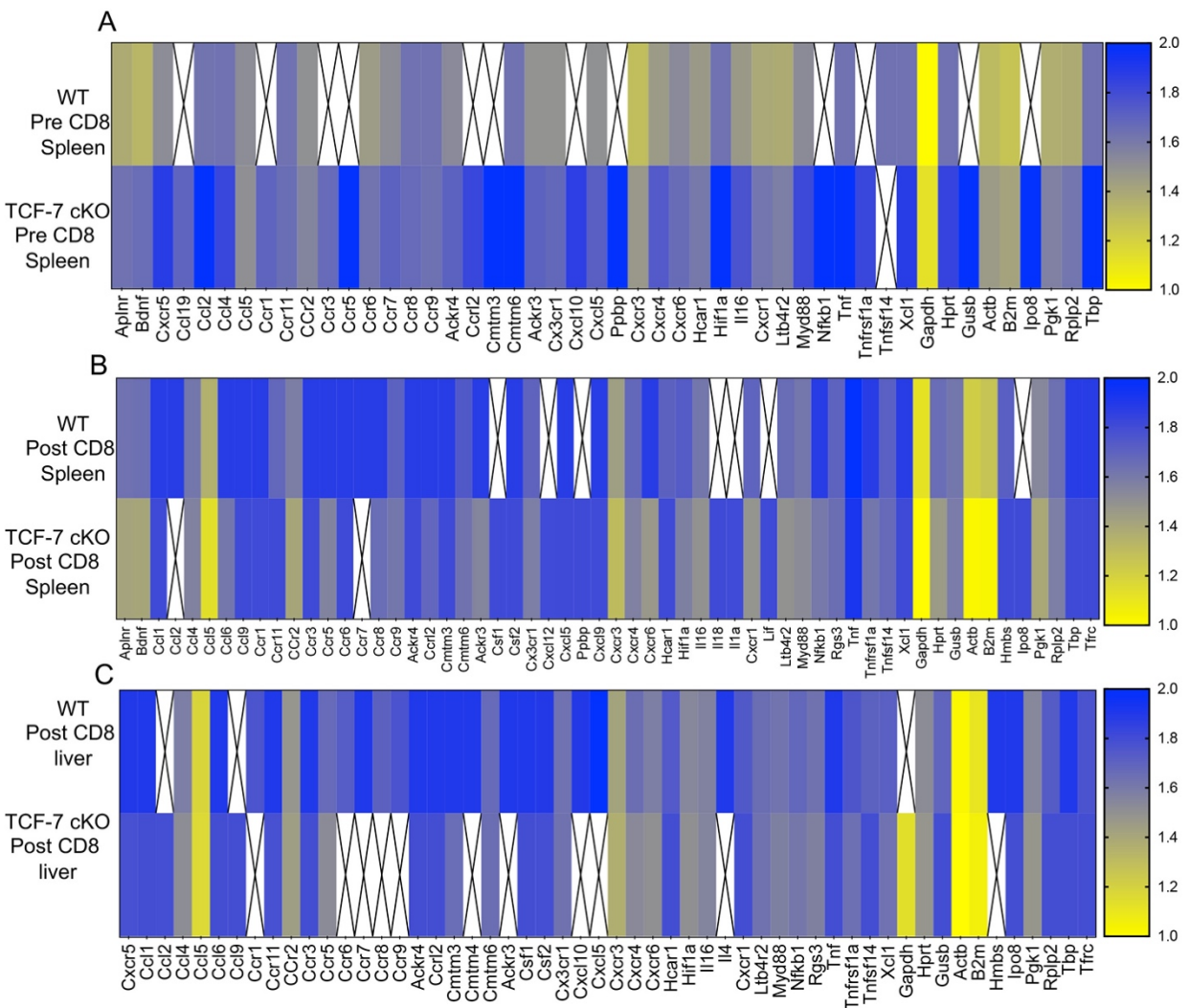
1077 **Supp.Fig.4. *TCF-7* controls chemokine and chemokine receptor expression of donor CD8 T**

1078 **cells during alloactivation.** As before, BALB/c mice were allotransplanted with BM and 1×10^6

1079 donor CD3 T cells from WT or *TCF-7* cKO mice (not mixed). Donor CD8 T cells were FACS-

1080 sorted from spleen of donor's pre-transplant, and from spleen and liver of recipients at day 7

1081 post-transplant. Cells were sorted into Trizol, then RNA was extracted using chloroform and

1082 converted to cDNA for qPCR analysis. cDNA was run on premade mouse chemokine/chemokine

1083 receptor assay plates, and results are displayed as heatmaps. Scales are shown at right, with fold

1084 change per gene compared to an 18S reference gene on each plate. White boxes with an "X"

1085 represent signals too low to detect or otherwise unreadable due to technical limitation/error. **(A)**

1086 Pre-transplant spleen donor CD8 T cells, **(B)** post-transplant spleen donor CD8 T cells, and **(C)**

1087 post-transplant liver donor CD8 T cells for WT versus *TCF-7* cKO donors. N=5 mice into one

1088 sample per condition**,** summary data shown.
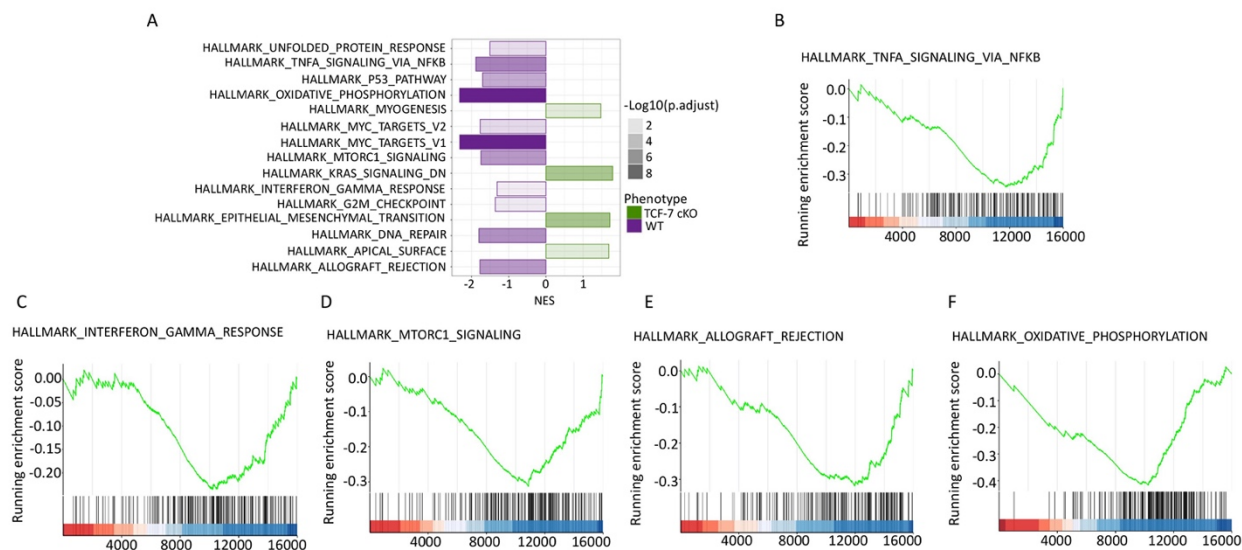
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1090 **Supp.Fig.5: Loss of *TCF-7* alters the enrichment of gene sets in pre-transplanted CD8+T**

1091 **cells. (A)** Bar plot of the altered pathways indentified in Gene set enrichment analysis (GSEA)

1092 by using Hallmark gene set collections of the Molecular Signatures Database (MSigDB) in pre-

1093   transplanted CD8 T cells (*TCF-7* cKO compared to WT). The normalized enrichment score

1094   (NES) for the pathway is defined as the peak score furthest from zero, with a negative NES

1095   meaning enrichment in the WT group. **(B)** GSEA plot for the

1096   "HALLMARK_TNFA_SIGNALING_VIA_NFKB" pathway comparing pre-transplanted CD8 T

1097   cells from *TCF-7* cKO to WT cells. The running enrichment score (ES) for the pathway is

1098   defined as the peak score furthest from zero, with a negative ES meaning enrichment in the WT

1099   group. **(C)** GSEA plot for the "HALLMARK_INTERFERON_GAMMA_RESPONSE" pathway

1100   comparing pre-transplanted CD8 T cells from *TCF*-7 cKO to WT cells. **(D)** GSEA plot for the

1101   "HALLMARK_MTORC1_SIGNALING" pathway comparing pre-transplanted CD8 T cells

1102   from *TCF-7* cKO to WT cells. **(E)** GSEA plot for the

1103   "HALLMARK_ALLOGRAFT_REJECTION" pathway comparing pre-transplanted CD8 T cells

1104   from *TCF-7* cKO to WT cells. **(F)** GSEA plot for the

1105   "HALLMARK_OXIDATIVE_PHOSPHORYLATION" pathway comparing pre-transplanted

1106   CD8 T cells from *TCF-7* cKO to WT cells.

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Top 20 pathways in GO-BP

Term ID	Term	Count	%	P Value	Fold Enrichment	FDR
GO:0007049	Cell cycle	141	5.73870574	2.34E-14	1.906511069	1.19E-10
GO:0015031	Protein transport	134	5.45380545	3.42E-13	1.879194277	8.74E-10
GO:0098609	Cell-cell adhesion	55	2.23850224	6.46E-10	2.415958527	9.33E-07
GO:0051301	Cell division	88	3.58160358	7.31E-10	1.953438125	9.33E-07
GO:0007067	Mitotic nuclear division	69	2.80830281	5.24E-09	2.068035126	5.35E-06
GO:0006915	Apoptotic process	114	4.63980464	4.40E-08	1.660422406	3.75E-05
GO:0006810	Transport	291	11.8437118	1.39E-07	1.325968496	1.01E-04
GO:0006974	Cellular response to DNA damage stimulus	86	3.5002035	8.53E-07	1.699956273	5.31E-04
GO:0016192	Vesicle-mediated transport	52	2.11640212	9.36E-07	2.026806693	5.31E-04
GO:0006783	Heme biosynthetic process	12	0.48840049	2.25E-06	5.243439176	0.0011491
GO:0002479	Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	15	0.61050061	2.85E-06	4.151056015	0.00132461
GO:0050852	T cell receptor signaling pathway	20	0.81400081	3.32E-06	3.255730208	0.00141253
GO:0006260	DNA replication	34	1.38380138	5.93E-06	2.294892756	0.00232917
GO:0002376	Immune system process	76	3.09320309	1.26E-05	1.647416486	0.00460664
GO:0043123	Positive regulation of I-kappaB kinase/NF-kappaB signaling	36	1.46520147	6.87E-05	2.005879416	0.02338981
GO:0008152	Metabolic process	84	3.41880342	1.21E-04	1.506214709	0.03848315
GO:0031663	Lipopolysaccharide-mediated signaling pathway	13	0.52910053	1.40E-04	3.481530851	0.04077673
GO:0006281	DNA repair	62	2.52340252	1.44E-04	1.618650773	0.04077673
GO:0008643	Carbohydrate transport	14	0.56980057	2.33E-04	3.141339687	0.06257663

1107

1108 **Supp.Fig.6. Related to Fig.7**. Top 20 GO-BP terms identified in DAVID functional annotation

1109 analysis of differentially expressed genes in post-transplanted samples. Count mean – number of

1110 genes involved in the Term, % - percentage of involved genes/ total genes in the Term.
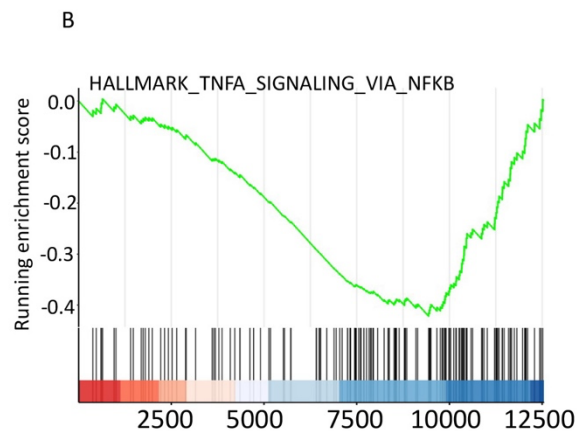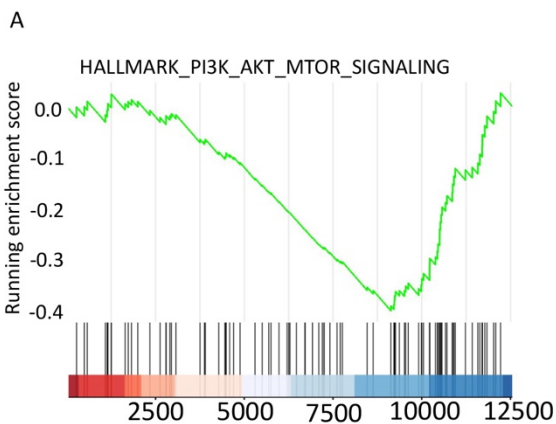
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1112 **Supp.Fig.7. Loss of *TCF-7* alters the Gene Set Enrichment Analysis (GSEA) of post-**

1113 **transplanted CD8+T cells. (A)** GSEA plot for the

1114 "HALLMARK_PI3K_AKT_MTOR_SIGNALING" pathway comparing post-transplanted CD8

1115 T cells from *TCF-7* cKO to WT mice. The running enrichment score (ES) for the pathway is

1116 defined as the peak score furthest from zero, with a negative ES meaning enrichment in the WT

1117 group. **(B)** GSEA plot for the "HALLMARK_TNFA_SIGNALING_VIA_NFKB" pathway

1118 comparing post-transplanted CD8 T cells from *TCF-7* cKO to WT mice. Again, the running

1119 enrichment score (ES) for the pathway is defined as the peak score furthest from zero, with a

1120 negative ES meaning enrichment in the WT group.

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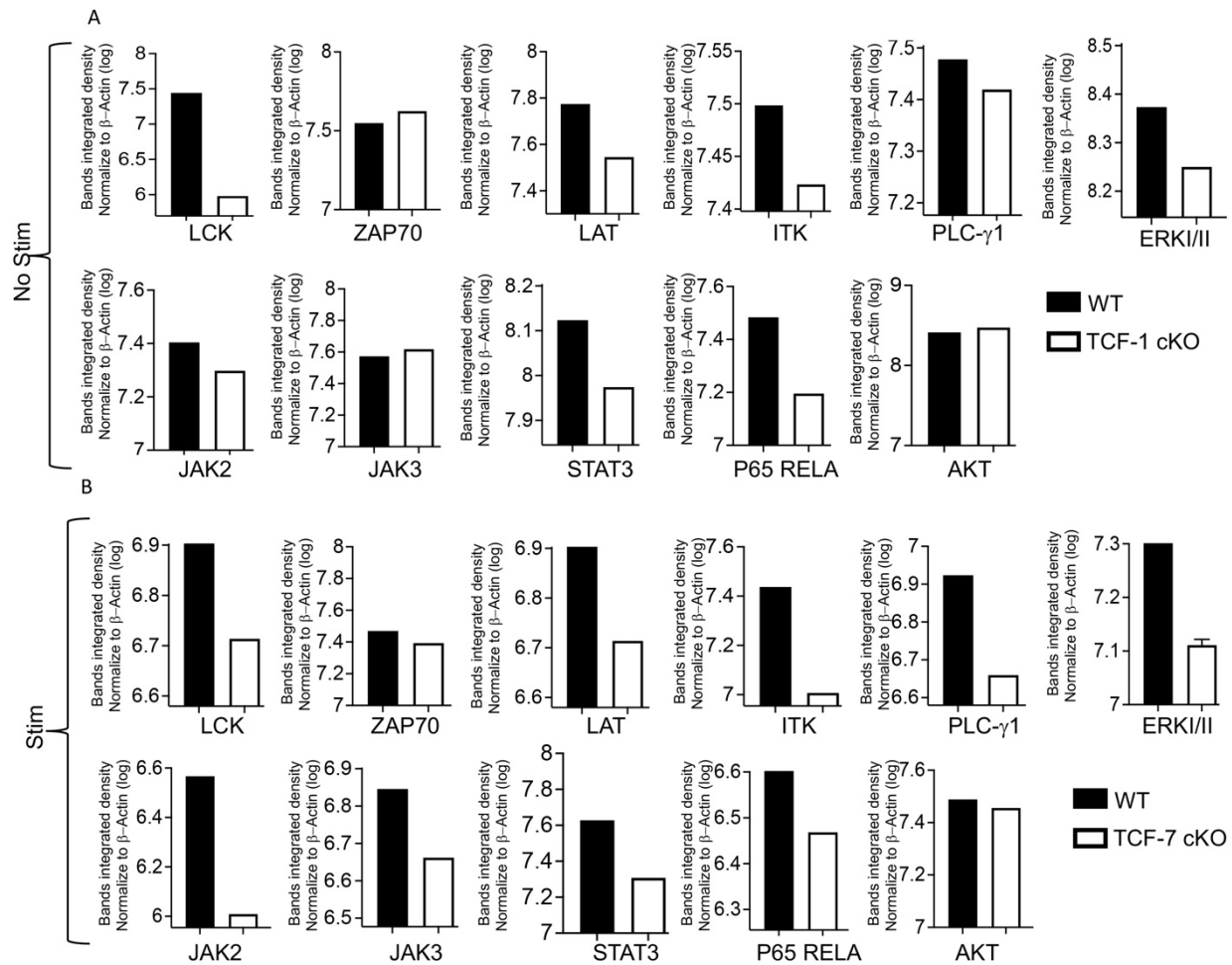
1122   **Supp.Fig.8. Related to Fig.8**. **Quantification of Western blot of TCR and JAK-STAT**

1123   **signaling.** **(A)** Comparison of the quantified bands integrated density normalized to β-actin for

1124   unstimulated CD8 T cells from *TCF-7* cKO and WT mice**. (B)** Comparison of the quantified

1125   bands integrated density normalized to β-actin for 10-minute-anti-CD3/CD28-stimulated CD8 T

1126   cells from *TCF-7* cKO and WT mice. All the western blots repeated at least three times and one

1127   representative of each protein and quantification is shown.

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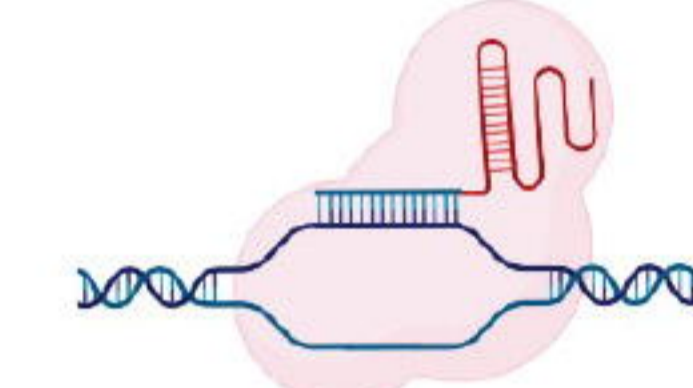
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1483

A

Days

Group1 Group2 Group1 Group3 Group1 Group4

6
10
15
24
28
32
46
53
67

✗ Death by GVL
✝ Death by GVHD

Photons/sec/cm2/sr.

TCF-1 cKO   WT   TCF-1 cKO   WT

● _TCD_BM Group 1
○ _TCD_BM+ A-20 Group 2
● _TCD_BM+ A-20+WT CD8 Group 3
◇ _TCD_BM+ A-20+TCF-7 cKO CD8 Group 4

B

Clinical score

****

Days Post transplantation

C

Weight (% of Initial)

****

Days Post transplantation

D

% Survival

****

Days Post transplantation

E

Log^{10} (photons/sec)

*

Days Post transplantation

A

B

C

D No stim

E Stim