

Generation and first characterization of TRDC-Knockout pigs lacking $\gamma\delta$ T cells

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1 **Abstract**

2 The TRDC-Locus encodes the T cell receptor delta constant region, one component of the
3 $\gamma\delta$ T cell receptor which is essential for development of $\gamma\delta$ T cells. In contrast to peptide
4 recognition by $\alpha\beta$ T cells, antigens activating $\gamma\delta$ T cells are mostly MHC independent and not
5 well characterized. Therefore, the function of $\gamma\delta$ T cells and their contribution to protection
6 against infections is still unclear. Higher numbers of circulating $\gamma\delta$ T cells compared to mice,
7 render the pig a suitable animal model to study $\gamma\delta$ T cells. Knocking-out the porcine TRDC-
8 locus by intracytoplasmic microinjection and somatic cell nuclear transfer resulted in healthy
9 living $\gamma\delta$ T cell deficient offspring. Flow cytometric analysis revealed that TRDC-KO pigs lack
10 $\gamma\delta$ T cells in peripheral blood mononuclear cells (PBMC) and spleen cells. The composition
11 of the remaining leucocyte subpopulations was not affected by the depletion of $\gamma\delta$ T cells.
12 Genome-wide transcriptome analyses in PBMC revealed a pattern of changes reflecting the
13 impairment of known or expected $\gamma\delta$ T cell dependent pathways. Histopathology did not
14 reveal developmental abnormalities of secondary lymphoid tissues. However, in a
15 vaccination experiment the KO pigs stayed healthy but had a significantly lower neutralizing
16 antibody titer as the syngenic controls.

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18

19 Introduction

20 The adaptive immune system is composed of three lymphocyte subsets, B cells, T cells
21 expressing the $\alpha\beta$ T cell receptor (TCR) and T cells expressing $\gamma\delta$ TCRs. The tripartite
22 organization of the lymphocytic immune system seems to be fundamental since it evolved
23 independently in jawed and jawless vertebrates¹. These cells generate their antigen specific
24 receptors using somatic V(D)J recombination, which enables them to recognize a vast
25 spectrum of different antigens. While the nature of antigen recognition by B cells and $\alpha\beta$ T
26 cells is well established, a unifying concept for $\gamma\delta$ T cell antigen recognition and function is
27 still pending. The majority of $\gamma\delta$ T cells are activated in an MHC-independent manner, which
28 is in striking contrast to MHC-restricted $\alpha\beta$ T cells. They can attack target cells directly
29 through their cytotoxic activity or indirectly through activation of other immune cells². Until
30 recently, $\gamma\delta$ T cells were thought to be simply innate immune cells with limited or redundant
31 functions³. The current view is that these cells complement many different players of the
32 immune system⁴, and it is becoming obvious that they represent a heterogenous population
33 of cells with important unique features in many infections, autoimmune diseases, allergies
34 and in immunoregulation. A wide range of $\gamma\delta$ T cell functions have been described in humans
35 and mice, including skin and mucosal epithelial wound repair, induction of tolerance,
36 cytotoxicity and the production of various cytokines that regulate immune responses⁵⁻⁸. The
37 increase in $\gamma\delta$ T cells following vaccinia virus infection, high vaccinia virus replication in $\gamma\delta$ T
38 cell knockout mice, and the selective lysis of $\gamma\delta$ T cells against vaccinia virus infected target
39 cells indicate some role for $\gamma\delta$ T cells during virus infections⁹⁻¹¹. To understand what they do
40 and what role they play in different challenging situations of the immune system, a knockout
41 animal model would be indispensable. A $\gamma\delta$ T cell deficient mouse model was successfully
42 established¹², however $\gamma\delta$ T cells show a remarkable degree of diversification in function,
43 anatomical localization and TCR usage that also differs largely between species¹³.
44 Therefore, care has to be taken when extrapolating results from one species to the other. In
45 humans and mice $\gamma\delta$ T cells constitute only 0.5–10% of T cells in peripheral blood but are
46 substantially enriched in epithelial tissues (e.g. in skin, lungs, intestine). A prominent

47 population of $\gamma\delta$ T cells in human blood, $V\gamma 9V\delta 2$ T cells which recognize phosphoantigens
48 (PAGs) do not exist in mice but were recently described in the new world camelid *Vicugna*
49 *pacos* (alpaca)¹⁴. Furthermore, while in human and mice a limited number of $\gamma\delta$ VDJ
50 cassettes exist, species like cattle, pigs and chicken have larger numbers of $\gamma\delta$ VDJ
51 cassettes and a high percentage of circulating $\gamma\delta$ T cells (20 - 50 %), thus called $\gamma\delta$ T cell-
52 high species in contrast to $\gamma\delta$ T cell-low species (humans and mice)¹⁵. In addition, in some
53 species the co-receptor WC1 family, which belong to the group B scavenger receptor
54 cysteine-rich molecules, is expanded¹⁶. WC1 receptors are exclusively expressed by $\gamma\delta$ T
55 cells and function as hybrid pattern recognition receptors and $\gamma\delta$ TCR co-receptors in cattle
56¹⁷. Bovine WC1 receptors can directly bind to pathogenic bacteria and transduce activation
57 signals to $\gamma\delta$ T cells, thus, $\gamma\delta$ T cells may be crucial for the control of certain systemic
58 infections¹⁷. Based on the remarkable lack of conservation between $\gamma\delta$ T cells of different
59 species, additional animal models are needed to understand the function of $\gamma\delta$ T cells and to
60 establish a unifying concept for their role during immune responses. In this report we
61 describe the generation of TRDC-KO pigs and their genetically identical wild type controls.
62 Since the number and quality of inbred pig strains are limited, the identity at the MHC locus
63 of the KO and control animals is of utmost importance for the comparison of the immune
64 response to infections. We did not observe any abnormalities in health and behavior of the
65 KO pigs under standard housing conditions. In addition, the immune system in KO pigs
66 developed normally, with the exception of the absence of $\gamma\delta$ T cells. The availability of a $\gamma\delta$ T
67 cells deficient animal model from a $\gamma\delta$ T cell-high species may add important new insights in
68 the role of this mysterious part of the adaptive immune system.

69

70 **Results**

71 **Intracytoplasmic microinjection of porcine *in vitro* derived zygotes with TRDC-**
72 **CRISPR/Cas and transfer to recipients**

73 After transfer of 35 microinjected embryos, both recipients (#695, #696) were determined
74 pregnant on day 25 after surgical embryo transfer. Sow 695 delivered 5 liveborn piglets on
75 day 112 of gestation and sow 696 gave birth to 9 liveborn piglets on day 114 of gestation
76 (Table 1). All piglets were healthy and showed no aberrations regarding birth weight
77 compared to age-matched counterparts or wild type littermates.

78 **Genotyping of TRDC-KO piglets**

79 Genomic DNA from tail tips was used for PCR-based detection of genetic modifications at
80 the TRDC locus in the piglets. The genomic DNA of the piglets originating from
81 intracytoplasmic microinjection of the pX330-TRDCEX4 #1 and #2 plasmids was employed
82 for PCR (Figure 1) and subsequent sequencing (Figure 2) to detect genetic modifications at
83 the TRDC gene. In total, two out of five piglets (40%) from sow 695 showed a biallelic 40 bp
84 deletion within exon 4 of the TRDC gene, while four out of nine piglets (44.4%) from sow 696
85 carried a biallelic 40 bp deletion. The remaining piglets were detected to be wild type. The
86 overall efficiency to generate a 40 bp biallelic deletion in the exon 4 of the TRDC gene was
87 42.9% (6/14).

88 **Transfection of CRISPR/Cas9 plasmids**

89 As an alternative to the intracytoplasmic microinjection approach, we employed somatic cell
90 nuclear transfer to produce genetically identical TRDC knockout pigs and syngeneic wild
91 type control pigs. Therefore, 3×10^6 cells in total were co-transfected with the plasmids
92 pX330-TRDCEX4#1 and #2 and subsequently seeded on three T25 flasks (25 cm²). After
93 reaching 70-80 % confluency, cells were detached by EDTA/Trypsin treatment and seeded
94 at a concentration of 7-10 cells per well on a 96-well plate. Individual cell clones were
95 analyzed by PCR. Cells with a biallelic deletion of the TRDC gene (- 40 bp) showed only the
96 lower band, while cell cultures with a mixed population of wild type, monoallelic and biallelic
97 genetically modified cells showed an upper wild type band at 499 bp and a lower band at 459
98 bp (Figure 3). Cell clones D12 (mixture) and H2 (almost pure lower band) were chosen as

99 donor cells for SCNT. D12 was chosen to produce both TRDC knockout and syngeneic wild
100 type control pigs.

101 **Somatic cell nuclear transfer of TRDC-KO embryos and syngeneic controls**

102 H2-derived cloned embryos were surgically transferred to two hormonally synchronized
103 recipients. Each of the two recipients (#8106 and #738) received 90 cloned embryos and
104 were detected to be pregnant on day 25 after embryo transfer. D12-derived cloned embryos
105 were also transferred to two recipients. One recipient (#8115) received 97 embryos, while the
106 second one received 96 embryos (#737). Again, both recipients were determined to be
107 pregnant by ultrasound scanning on day 25 after embryo transfer (Table 2). All recipients
108 were allowed to go to term and delivered in total 9 healthy liveborn (9/17:52.9%) and 8
109 stillborn piglets (8/17:47.1%). The stillborn piglets did not show any abnormalities but were
110 not checked for deletions in the exon 4 of the TRDC gene.

111 PCR analysis of the cloned piglets revealed that 4 out of the 7 piglets (57.1%) originating
112 from the mixed cell clone D12 (737-1-7) carried a biallelic 40 bp deletion of the TRDC gene,
113 while the remaining 3 piglets remained wild type (42.9%). The two piglets (738-1, -2)
114 originating from cell clone H2 carried a biallelic 40 bp deletion of the TRDC gene, as
115 expected (Figure 4).

116 **Fitness and viability of TRDC-knockout pigs**

117 The TRDC knockout pigs did not differ to syngenic wild type control animals and age-
118 matched wild type pigs in our facility in regard of their health status and growing performance
119 (Figure 5). TRDC-KO pigs are kept for 2 years in our facility under standard conditions and
120 never showed any health impairment.

121 **FACS analysis of TRDC-knockout blood and spleen cells**

122 To determine the effect of the 40 bp deletion in the TRDC gene on lymphocyte composition
123 in TRDC-KO pigs we performed flow cytometry analysis of PBMC and spleen cells from 8 wt
124 pigs and 5 TRDC-KO pigs. We used two mAb that specifically detect independent antigens
125 of porcine $\gamma\delta$ T cells. One binds to the δ -chain of the T cell receptor and the other to the CD3

126 antigen of $\gamma\delta$ T cells. In average 20 % (ranging from 8 % – 47 %) of peripheral blood T cells
127 were double-stained by these mAbs in wild type pigs while no double-stained cells were
128 detected in TRDC-KO pigs, indicating that no $\gamma\delta$ T cells were present in peripheral blood of
129 TRDC-KO pigs (Figure 6A). In addition, we isolated spleen cells from wild type and TRDC-
130 KO pigs and analyzed the content of $\gamma\delta$ T cell populations. Typically, in wild type pigs the
131 majority (80 %) of $\gamma\delta$ T cells in the blood belong to the CD2 negative phenotype, while in the
132 spleen the majority (66 %) of $\gamma\delta$ T cells are CD2 positive. No $\gamma\delta$ CD3 positive cells of either
133 type were detected in the spleen of TRDC-KO pigs (Figure 6B). These data indicate that no
134 $\gamma\delta$ T cells developed in TRCD-KO pigs. The majority of $\gamma\delta$ T cells in pigs are CD4 and CD8
135 negative, reflected by minimal numbers of CD4/CD8 double negative T cells in TRCD-KO
136 pigs (Figure 6C). Comparing the numbers of various lymphocyte subpopulations between
137 TRDC-KO pigs and wild type pigs we did not find significant differences (Figure 6D). These
138 results indicated that the absence of $\gamma\delta$ T cells had no major impact on the development of
139 other lymphocyte populations.

140 **Comparison of gene expression profiles of TRDC-KO versus control animals via** 141 **microarray analysis**

142 Since we did not detect a major influence on the composition of lymphocyte subpopulations
143 in the peripheral blood by the complete loss of $\gamma\delta$ T cells, we asked whether the absence of
144 $\gamma\delta$ T cells has an impact on the transcriptional profile of the remaining lymphocyte
145 population. Thus, we performed microarray analysis of PBMC isolated from wild type pigs
146 and TRDC-KO pigs. Only 0.7 % of genes were regulated more than 2-fold (0.4 %
147 upregulated and 0.3 % downregulated) in PBMC of TRDC-KO pigs (Figure 7A). Hierarchical
148 clustering identified gene clusters with a significant expression level in either the wild type or
149 the TRDC-KO pigs, only these genes were further analyzed (Figure 7B). A high portion of
150 down-regulated genes are known to be preferentially expressed by $\gamma\delta$ T cells, such as δ T
151 cell receptor genes, GATA3, WC1.1, SOX13, IL-1R and IL-18R. Genes which were
152 upregulated in TRDC-KO pigs encompass genes which are preferentially expressed by
153 granulocytes and monocytes in human PBMC like Zyxin, CXCL8 and CXCL2. Thus, the

154 transcriptional profile of PBMC in TRDC-KO pigs reflect the loss of $\gamma\delta$ T cells and as a result
155 the proportional increase of non-lymphoid cells.

156 **No differences between wild type and TRDC-KO pigs were detected by gross**
157 **pathological, histological and immunohistological examination of lymphoid tissues**

158 We wondered if the development of lymphoid tissues was influenced by the absence of $\gamma\delta$ T
159 cells. At autopsy the size and morphology of all lymphoid tissues did not differ between wild-
160 type or knockout pigs. Further, neither the detailed histopathologic evaluation on H&E
161 stained slides nor the application of T-cell (CD3) and B-cell (CD20) markers revealed
162 differences in the overall architecture, distribution and quantity of the immune cells labelled.
163 Representative slides of the thymus, ileal Peyer's patches and tonsil are shown in Figure 8
164 (A-R). Both mAbs directed against the specific $\gamma\delta$ T cell-antigens (PGBL22A, PPT16) cross
165 react on tissue slides with cytoplasmic structures of an unknown cell type, resulting in
166 unspecific reactions. Thus both antibodies we had at hand, were unfortunately not suited for
167 the use in immunohistology on cryosections.

168 **Possible role of $\gamma\delta$ T cells in vaccine-mediated protection**

169 As a first approach towards testing the immunological competence of the TRDC-KO pigs we
170 decided to vaccinate the animals with a commercial vaccine against the pestivirus classical
171 swine fever virus (CSFV). The vaccine contains a highly attenuated live virus of the so-called
172 C-strain type. In normal pigs, the vaccine is known to be completely apathogenic but very
173 effective with regard to induction of a protective immunity against CSFV field virus infection¹⁸⁻
174 ²¹. Three knockout pigs [group 1, numbers 1314, 1318, 1319] and two syngenic wild type
175 controls [group 2, numbers 1315, 1320] were vaccinated at day 0 with the commercial live
176 virus vaccine via the intramuscular route as recommended by the supplier. Rectal
177 temperature of the animals was recorded daily starting 10 days before vaccination. The
178 animals were monitored daily for general health status. Neither in group 1 nor 2 any signs of
179 disease were detected during the observation period. Body temperatures of all animals
180 remained in the normal range. Blood for serum production was taken on day 0 and on day 21

181 p.i. The sera were tested for the presence of neutralizing antibodies against the homologous
182 CSFV C-strain. As expected, the results were negative for all animals at day 0, proving that
183 they had not had any contact to CSFV antigens before. In contrast, neutralization titers were
184 recorded on day 21 for all animals (Figure 9). The values were significantly higher for the wild
185 type pigs compared to TRDC-KO (P value = 0.0428).

186 Discussion

187 Mice deficient for $\gamma\delta$ T cells have been established almost 30 years ago²². In the meantime,
188 they have proven to be extraordinary helpful to elucidate functions of $\gamma\delta$ T cells. In particular,
189 the role and contribution of certain murine $\gamma\delta$ T cell compartments during infections with viral,
190 bacterial and parasitic pathogens could be defined using these mice²³⁻²⁵. Taken into account
191 that $\gamma\delta$ T cell populations can differ largely between species it is of considerable importance
192 to have $\gamma\delta$ T deficient animals from additional species. One major variation of $\gamma\delta$ T cell
193 compartments between species is the amount of circulating $\gamma\delta$ T cells. The proportion of
194 circulating $\gamma\delta$ T cells is surprisingly high in artiodactyls (e.g. cattle, sheep and pigs) and
195 chicken¹⁵. Thus, the animal model described in the present report is the first of a $\gamma\delta$ T cell-
196 high species. Pigs with a biallelic knockout of the TRDC gene were efficiently produced
197 either by intracytoplasmic microinjection or somatic cell nuclear transfer using modified cells
198 as donor cells. While healthy offspring could be obtained after embryo transfer of
199 microinjected zygotes, somatic cell nuclear transfer (SCNT) resulted in 47.1 % stillborn
200 piglets. As healthy TRDC knockout piglets were obtained by both techniques, we conclude
201 that the relatively high number of stillborn piglets was associated with SCNT and false or
202 incomplete reprogramming of the donor cell nucleus²⁶⁻²⁸. TRDC knockout pigs developed
203 normally and reached sexual maturity. After breeding TRDC deficient pigs, all offspring
204 carried a monoallelic knockout of the TRDC gene as expected. Though mosaicism cannot be
205 excluded in the pigs originating from intracytoplasmic microinjection²⁹, we found the 40 bp
206 deletion in all tissues analyzed (skin, heart, liver, kidney, muscle), rendering mosaicism
207 unlikely. Besides, no off-target events could be detected in the 10 most possible genomic
208 sites in microinjected and cloned offspring (data not shown). As previously observed in

209 Tcrd-/- mice no $\gamma\delta$ T cells remain in the TRDC-KO pigs while the studies suggest the
210 presence of a normal $\alpha\beta$ T cell repertoire in the periphery of the $\gamma\delta$ T cell deficient pigs²².
211 Furthermore, microarray analysis did not indicate that there is a major impact on the
212 transcriptome of PBMC by the loss of $\gamma\delta$ T cells. These observations may explain why the
213 immune phenotype of TRDC-KO pigs is surprisingly mild. The vaccination experiment
214 described above was conducted with a commercial vaccine containing a live virus that is
215 known to be completely apathogenic in wild type pigs¹⁸⁻²¹. We have chosen this approach to
216 check whether the knockout pigs are hampered in a way that they cannot control a virus
217 infection at all, even when the infecting virus is highly attenuated. In fact, we saw no signs of
218 disease in the vaccinated pigs which could be explained in different ways, namely that the so
219 far unknown attenuation principle of CSFV C-strain is sufficient even in immunologically
220 compromised animals, that the loss of $\gamma\delta$ T cells has only mild effects on virus control by the
221 immune system or a mixture of both reasons. The results of the antibody neutralization test
222 showed that the knockout pigs were able to mount a measurable antibody response to an
223 infecting virus, but the titers were lower than in the wild type animals. Even though the
224 statistical significance of this result is only low due to the small number of animals tested, it
225 represents a first indication for a reduced immunological competence of the knockout
226 animals. This may indicate that different outcomes of infections are due to the missing
227 contribution of $\gamma\delta$ T cells during the immune response not to other deficiencies of the immune
228 system. This point will have to be followed up using different infection and challenge
229 systems. There are various reports pointing to a protective role of $\gamma\delta$ T cells against
230 important pig pathogens such as African swine fever virus^{30,31}, Foot and mouth disease
231 virus^{32,33}, Mycobacterium bovis³⁴, Porcine respiratory and reproductive syndrome virus^{35,36},
232 and Influenza A virus³⁷. A specific role of $\gamma\delta$ T cells has not been definitively proven so far,
233 but with the TRDC-KO pigs at hand, the large variety of swine pathogens displaying different
234 ways of pathogenesis and interplay with the hosts immune system can be tested to finally
235 elucidate the role of $\gamma\delta$ T cells in immunological defense against infectious agents. The fact

236 that some of these pathogens are zoonotic and responsible for severe human diseases
237 render this approach even more attractive.

238 **Material and Methods**

239 **Animals**

240 German Landrace pigs served as recipient animals for genetically modified embryos
241 derived by somatic cell nuclear transfer (SCNT).

242 **CRISPR/Cas vector and single-guide RNA**

243 The CRISPR/Cas9 system was used to induce defined deletions within the exon 4 of the
244 porcine TRDC gene (Ensembl transcript: ENSSSCT00000026772). Guide RNAs (gRNAs)
245 were designed using the web-based design tool *CRISPOR* (<http://crispor.tefor.net/>) (Fig. X).
246 Target sequences were analyzed via BLAST to reduce the probability for off-target events.
247 Two oligo duplexes including the target sequence (TRDCEX4#1: 5' -CAC CGT GAT GTC
248 TGT CAC AGT GCT T-3' and TRDCEX4#2: 5'-CAC CGG CAG TCA AGA GAA AAT TGA-
249 3') and BbsI overhangs were designed, and each gRNA was cloned into a linearized
250 CRISPR/Cas9 vector pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42230)³⁸.
251 The final plasmids pX330-TRDCEX4#1 and pX330-TRDCEX4#2 were then used for
252 intracytoplasmic microinjection into IVF zygotes and for transfecting porcine fetal fibroblasts.

253 **Culture and lysis of primary cell cultures from wild type fetuses**

254 Porcine fibroblasts were isolated from ear tissue of wild type piglets and cultured in
255 Dulbecco's modified Eagle's medium (DMEM) with 2 % penicillin/streptomycin, 1 % non-
256 essential amino acids and sodium pyruvate and 30 % fetal calf serum (FCS) (Gibco, 10270-
257 106). After the first passage the antibiotic concentration was reduced to 1%.

258 **Transfection of CRISPR/Cas9 plasmids**

259 In total, 3×10^6 cells were transfected when they reached 70-80% confluency. The two
260 CRISPR/Cas9 plasmids (pX330-TRDCEX4#1+2) were co-transfected (at a final
261 concentration of 5 $\mu\text{g}/\mu\text{l}$) into porcine fibroblasts by electroporation (NeonTM Transfection

262 System, ThermoFisher Scientific) to test the efficacy of the plasmids to induce a 40bp
263 deletion at the targeted locus (Figure X). Electroporation conditions were as follows: 1350 V,
264 20 mm, and two pulses. After lysis of transfected cells in cell lysis buffer followed by ethanol
265 extraction, the DNA was analyzed using TRDC specific primer (TRDCEx4-2F: 5'-
266 CTGGGTGTAAGTAGCAGCCT-3' and TRDCEx4-2R: 5'-ACACGAGTTTTGAGTCTGGC-3').
267 The purified 499bp PCR product (10 ng/μl) (Invisorb® Fragment CleanUp – Startec) was
268 Sanger sequenced to detect mutations at the target site. To produce TRDC-KO cell clones
269 for serving as donor cells in SCNT, cells were trypsinized and subsequently diluted to a final
270 concentration of 7-10 cells per well.

271 **In-Vitro-Fertilization and In-Vitro-Maturation.**

272 In-vitro-maturation of porcine oocytes was performed as previously described³⁹. Briefly,
273 porcine oocytes were collected from ovaries derived from slaughterhouse and matured for 40
274 hours in FLI medium. For in vitro fertilization, frozen boar semen from a fertile landrace boar
275 was thawed for 30 seconds in a water bath (37 °C). The sperm motility was microscopically
276 checked (Olympus, BH-2). After washing with Androhep® Plus (Minitube) and centrifugation
277 for 6 minutes at 600 g, approx. 75 to 100 sperm per oocyte (depending on semen capacity)
278 were used for fertilization (no sexed sperm were utilized for fertilization). After four hours of
279 co-incubation, the fertilized oocytes were cultured in porcine-zygote-medium (PZM-3
280 medium).

281 **Intracytoplasmic Microinjection**

282 Plasmids were prepared in 10 mM Tris-HCl pH 7.6 and 0.25 mM EDTA pH 8.0, and
283 backfilled in glass injection capillaries and diluted to a final concentration of 2.5 ng/μl.
284 Individual zygotes were fixed by suction to a holding pipette. The plasmids pX330-
285 TRDCEX4#1 and pX330-TRDCEX4#2 were intracytoplasmically co-injected into IVF-
286 produced zygotes derived from oocytes collected from slaughterhouse ovaries 20 hours after
287 fertilization. To this end, approx. 10 pl plasmid solution was injected with a pressure of 600
288 hPa into IVF-produced zygotes (FemtoJet, Eppendorf). The injected zygotes were cultured in

289 PZM-3 medium at 39 °C, 5 % CO₂ and 5 % O₂. At day 5, when embryos had reached the
290 morula/blastocyst stage, 35 embryos were surgically transferred into each of the two
291 recipients.

292 **Somatic cell nuclear transfer**

293 SCNT was performed as previously described⁴⁰. Fetal fibroblasts transfected with px330-
294 TRDCEx4#1 and px330-TRDC-Ex4#2 targeting the exon 4 of the porcine TRDC gene were
295 used as donor cells. In order to produce syngeneic wild type control pigs simultaneously, we
296 chose cell clone D12 that gave two bands with a 50:50 ration on the PCR gel (Fig. 3) and cell
297 clone H2 that gave an almost clean -40bp PCR band. In total, 90 H2-derived one- to two-cell
298 embryos were surgically transferred into each of two hormonally synchronized German
299 Landrace gilts (7 to 9-months old). For D12-derived embryos, 97 and 96 one- to two-cell
300 embryos were surgically transferred into two hormonally synchronized German Landrace
301 gilts mg/day/gilt Altrenogest (Regumate® 4mg/ml, MSD Germany) for 12 days, followed by
302 an injection of 1,000 IU PMSG (pregnant mare serum gonadotropin, Pregmagon®, IDT
303 Biologika) on day 13 and induction of ovulation by intramuscular injection of 500 IU hCG
304 (human chorion gonadotropin, Ovogest®300, MSD Germany) 72 h after PMSG
305 administration. The surgical embryo transfer was performed the day after the hCG
306 administration.

307 **Genotyping of transfected cells and TRDC-KO pigs**

308 Genomic DNA of the pigs was extracted from tail tips. Transfected cells and tail tips were
309 lysed in cell lysis buffer (10%SDS, Proteinase K (20mg/ml), 10xPCR Buffer, aqua dest.) and
310 purified by ethanol extraction. The DNA concentration was determined using the NanoDrop™
311 (Kikser-Biotech) system. For genotyping the pigs, polymerase chain reaction (PCR) was
312 employed using specific primers (TRDCEx4-2F: 5'-CTGGGTGTAAGTAGCAGCCT-3' and
313 TRDCEx4-2R: 5'-ACACGAGTTTTGAGTCTGGC-3') flanking a 499 bp segment of the TRDC
314 gene (Fig. X). PCR amplification was performed in a total volume of 50 µl: 20 ng DNA, 0.6
315 µM reverse and forward primer, 1.5 mM MgCl₂, 0.2 mM dNTPs and 1.25 U *Taq* Polymerase.

316 Cycling conditions were as follows: 32 cycles with denaturation at 94°C for 30 sec, annealing
317 at 60 °C for 45 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 minutes.
318 The standard conditions for gel electrophoresis were set up to 80 V, 400 mA and 60 min
319 using a 1 % agarose gel. The PCR-product was purified (Invisorb®Fragment CleanUp-Kit,
320 Startec) and Sanger sequenced.

321 **Allele-specific sequencing of the TRDC gene of cloned piglets**

322 The PCR product of the targeted region within exon 4 of the TRDC gene was subcloned into
323 the pGEM-T Easy Vector system (Promega, Germany) in accordance with the
324 manufacturer’s protocol and then transformed into XL10 bacteria. Bacteria were plated on
325 Ampicillin containing Agar dishes (100 µg/ml) and allowed to grow for 16 hours. For each
326 piglet, six colonies were picked and sequenced by using the T7prom primer.

327 **FACS analysis of TRDC-knockout blood and spleen cells**

328 PBMC were isolated by density gradient centrifugation using Lympholyte®-Mammal Cell
329 Separation Media (Cedarlane, Canada). Spleen cells were isolated by generation of a single
330 cell suspension and erythrocyte lysis with ammonium chloride. For flow cytometry 1×10^5 cells
331 were incubated with primary antibodies followed by an incubation with secondary antibodies.
332 The following primary antibodies were used anti-TCR $\gamma\delta$ (PGBL22A, IgG1; PPT16, IgG2b),
333 anti-CD2 (MSA4, IgG2a), anti-CD3 (PPT3, IgG1), anti-CD4 (74-12-4, IgG2b) and anti-CD8
334 (11/295/33, IgG2a). Detection of binding primary antibodies was performed using anti-mouse
335 IgG1-PE, anti-mouse IgG2a-FITC, anti-mouse IgG2b-APC. All incubations were done in acid
336 buffer at 4°C and dead cells were excluded by adding propidium iodide (PI) (dilution 1:1000
337 in acid buffer) to the cells before measurement. Flow cytometry was performed with the
338 MACSQuant Analyser and the “MACS Quantify” software.

339 **Transcriptome analysis**

340 RNA from isolated PBMC was extracted using TRIzol® Reagent (Thermo Fisher Scientific).
341 RNA from 4 TRDC-knockout pigs and 2 wild type pig were pooled, respectively. Pools were

342 used to assess the quality of the RNA using an agilent bioanalyser. Labeled fragmented
343 single-stranded cDNAs (ss-cDNA) were synthesized by using purified total RNA (100–
344 500 ng) as template following Affymetrix WT PLUS Labeling Assay protocols. Porcine Gene
345 1.1 ST Arrays (Affymetrix, Santa Clara, CA, USA) were hybridized to the biotinylated ss-
346 cDNA targets. After 20 h of hybridization at 48 °C, arrays were washed by a fluidics station
347 and then scanned by an imaging station in a GeneAtlas System (Affymetrix, Santa Clara,
348 CA, USA). After scanning, the intensity data (CEL files) of Porcine Gene 1.1 ST arrays
349 (Affymetrix) were extracted from the image data (DAT files) by the Affymetrix Command
350 Console Software Version 1.4, and then normalized and analyzed by the Affymetrix
351 Transcriptome Analysis Console (TAC) Software 4.0 for gene expression profiles and DEGs.
352 The DEGs were selected by a cutoff of fold change >2.

353 **Autopsy, Histology and immune fluorescence**

354 Full autopsy was performed on all pigs. Samples of the thymus, tonsil, spleen, femoral bone
355 marrow, ileal Peyer's patches, and lymph nodes (mandibular, tracheobronchial, cecal,
356 popliteal) were immersion-fixed in neutral buffered, 10% formalin for at least 72 hours, and
357 additional samples were snap frozen in liquid nitrogen.
358 Formalin-fixed tissues were trimmed, embedded in paraffin, cut in 2 µm sections and stained
359 with hematoxylin and eosin (H&E) according to standardized procedures. For
360 immunohistochemistry (IHC), consecutive slides were mounted on adhesive glass slides,
361 dewaxed in xylene, followed by rehydration in descending graded alcohols. Endogenous
362 peroxidase was quenched with 3% H₂O₂ in distilled water for 10 minutes at room
363 temperature (RT). Heat induced antigen retrieval was performed in a decloaking chamber for
364 10 minutes at 110°C in 10mM citrate buffer (pH 6). Nonspecific antibody binding was blocked
365 with 1:2 diluted goat normal serum in Tris-buffered saline (TBS) for 30 minutes at RT. A
366 polyclonal rabbit anti-CD3 (#A0452, diluted 1:100 in TBS, Dako Agilent, Santa Clara, CA,
367 USA) or rabbit anti-CD20 (#RB-9013-P1, diluted 1:200 in TBS, Thermo Fisher Scientific,
368 Waltham, MA, USA) was applied over night at 4°C. A secondary biotinylated goat-anti rabbit
369 antibody was used (#BA-1000, diluted 1:200 in TBS, Vector Laboratories, Burlingame, CA,

370 USA) for 30 minutes at RT. The red-brown antigen labelling was developed by application of
371 avidin-biotin-peroxidase complex (ABC) solution (Vectastain ABC Kit, #PK 6100, Vector
372 Laboratories), followed by exposure to 3-amino-9-ethylcarbazole substrate (AEC, Dako,
373 Agilent, Santa Clara, CA, USA). Sections were counter-stained with Mayer's hematoxylin,
374 dehydrated in ascending graded alcohols, cleared in xylene, and coverslipped. All slides
375 were digitized using the Aperio CS2 slide scanner (Leica Biosystems Imaging Inc., CA, USA)
376 and image files were generated using the NDP.view2 Software (Hamamatsu Photonics,
377 Hamamatsu City, Japan). The establishment of immunofluorescence double-labelling using a
378 polyclonal rabbit anti-CD3 and a monoclonal mouse anti-TCR $\gamma\delta$ /PGBL22A antibody on snap
379 frozen lymphoid tissues failed (not details shown). All examinations were performed by a
380 board-certified pathologist (DipIECVP).

381 **Vaccination experiment**

382 Five pigs at an age of 3 months were included in a vaccination study. Three of these animals
383 had a $\gamma\delta$ knockout phenotype (numbers 1314, 1318, 1319 (738-1, 737-4, 737-5) = group 1]
384 and two represented syngenic wild type controls [group 2, numbers 1315, 1320 (737-1, 737-
385 6)]. The two groups were kept in separate rooms. Vaccination was done after 14 days of
386 acclimatization with a commercial life CSFV vaccine (Pestiffa CL, Boehringer Ingelheim
387 Vetmedica, Ingelheim, Germany) according to the providers' recommendation via the
388 intramuscular route into the muscle brachiocephalicus. After vaccination, the animals were
389 monitored daily. Rectal temperatures were recorded daily from -10 dpv to 24 dpv. Blood
390 samples for serum production were taken on days 0 dpv and 21 dpv. Determination of
391 neutralizing antibody levels was done as essentially as described before^{41,42}

392 **Declarations**

393 **Ethics approval and consent to participate**

394 Animal experiments were approved by the supervisory authorities (LAVES, AZ 33.19-42502-
395 04-17/2532 and LALLF, 7221.3-2-042/17) and conducted in compliance with the German
396 animal welfare law, the German guidelines for animal welfare and the EU Directive

397 2010/63/EU. All experiments were performed in accordance with relevant guidelines and
398 regulations.

399 **Consent for publication**

400 Not applicable.

401 **Availability of data and materials**

402 Not applicable

403 **Competing interests**

404 The authors declare that they have no financial and non-financial competing interests.

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407 the design of the study and collection, analysis, and interpretation of data and in writing the
408 manuscript.

409 **Authors' Contributions**

410 B.P., R.K. and G.M. conceived and designed the study and carried out data analysis as well
411 as writing and revising the manuscript. B.P., A.F., P.H., R.B. and A.L.-H. established the
412 TRDC-KO pigs, T.H.D., A.B. and R.G.U. performed experiments and data analysis.

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415 Freja Pfirske, Gabriele Czerwinski and Maren Ziegler for excellent technical assistance.

416

417 **Figures**

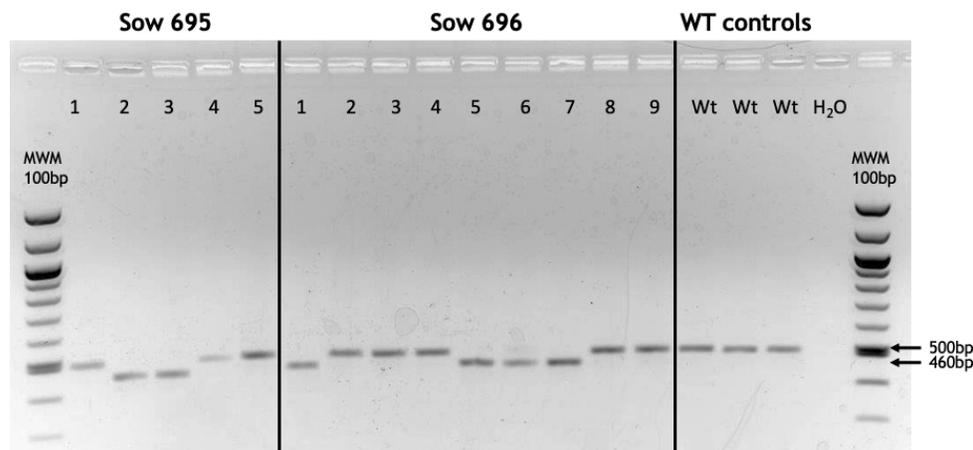
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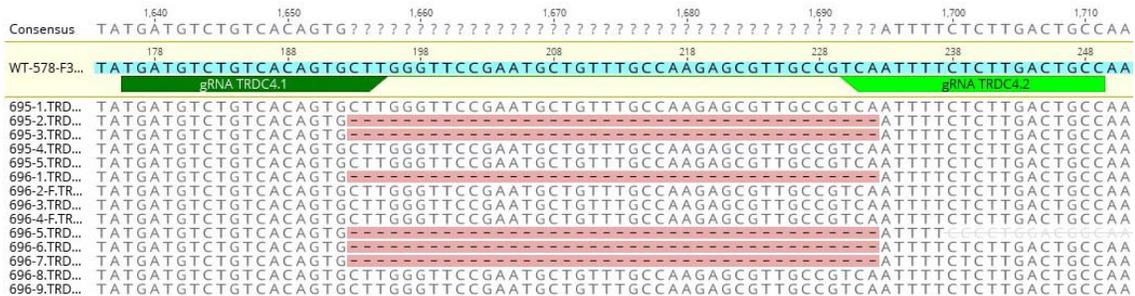
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426 **Figure 1: PCR-Analysis of the porcine TRDC locus in samples from pigs generated by**
 427 **intracytoplasmic microinjection.** Pigs 695-2, -3 and 696-1, -5, -6, -7 display a lower band
 428 of about 460 bp in contrast to wild type pigs (500 bp) indicating a 40 bp deletion caused
 429 application of the CRISPR/Cas9 construct.

430



431

432 **Figure 2: Sequencing data of pigs generated by intracytoplasmic microinjection of the**
 433 **CRISPR/Cas9 construct targeting the porcine TRDC-locus.** Sequencing results confirmed
 434 the PCR results (Fig.1) that pigs 695-2, -3 and pigs 696-1, -5, -6, -7 carry a homozygous
 435 knockout of the TRDC locus consisting of a clear 40 bp deletion. For each piglet, six samples
 436 were sequenced. None of the piglets were monoallelic knockouts. (The binding horderes of
 437 the guide RNAs are indicated by green arrows).

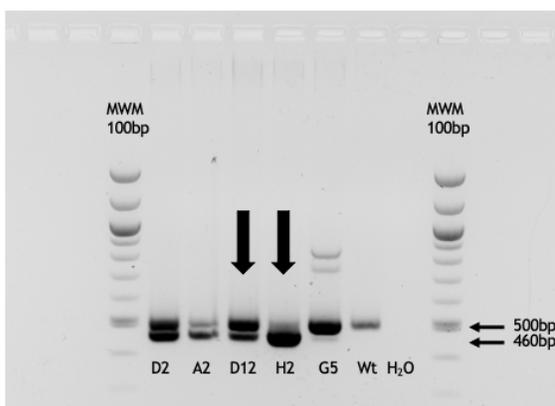
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445 **Figure 3: PCR analysis of the TRDC locus of CRISPR/Cas9 transfected cells.** The lower
446 band of 460bp indicates a 40bp deletion caused by the CRISPR/Cas9 construct. Cell clone
447 D12 displayed two bands of 500 and 460bp of size which indicates a mixed population of
448 cells consisting of TRDC-knockout cells, wild type cells and/or cells with a monoallelic
449 knockout of the TRDC locus. In contrast, cell clone H2 shows a single band of 460 bp
450 indicating an almost pure population of TRDC knockout cells.



451

452 **Figure 4: Sequencing results of the cloned liveborn TRDC knockout pigs.** Pig 737-4 differed
453 regarding the genetic modification compared to the other TRDC knockout pigs with a larger
454 deletion encompassing 45bp, indicating its origin from a separate cell subclone. The binding
455 sequences of the guide RNAs are indicated by red arrows.

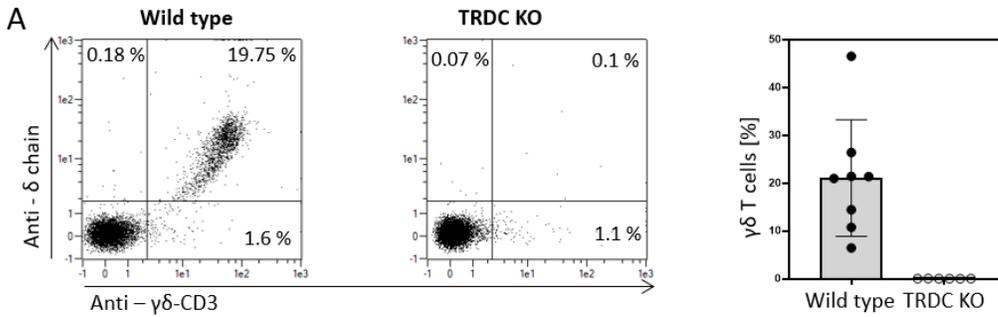
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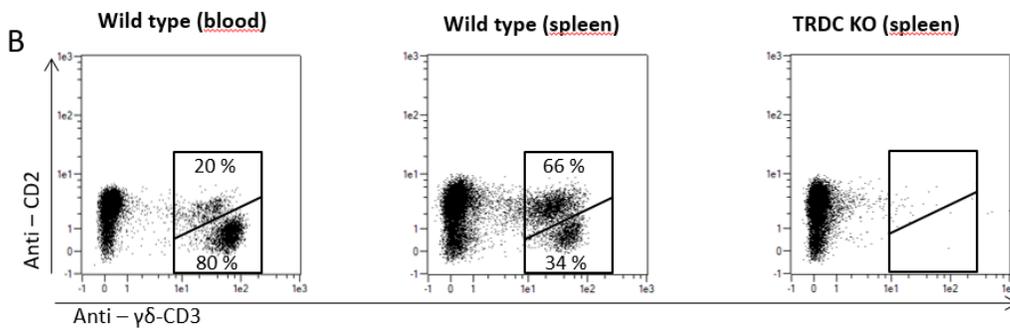
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458 **Figure 5:** Three TRDC-knockout pigs from intracytoplasmic microinjection.

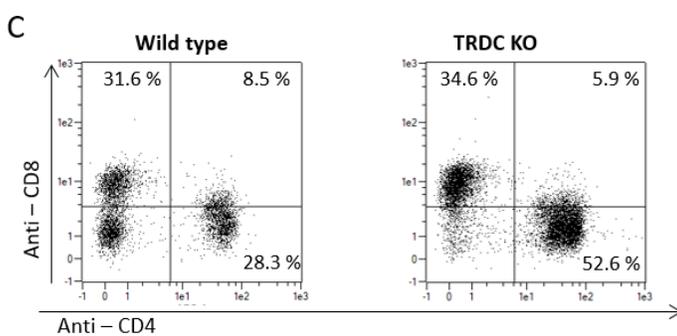
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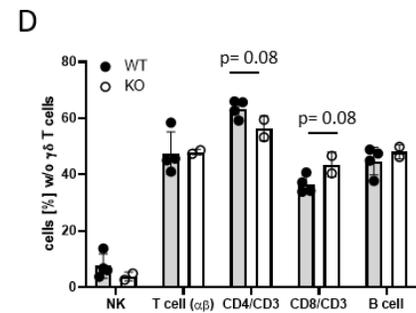
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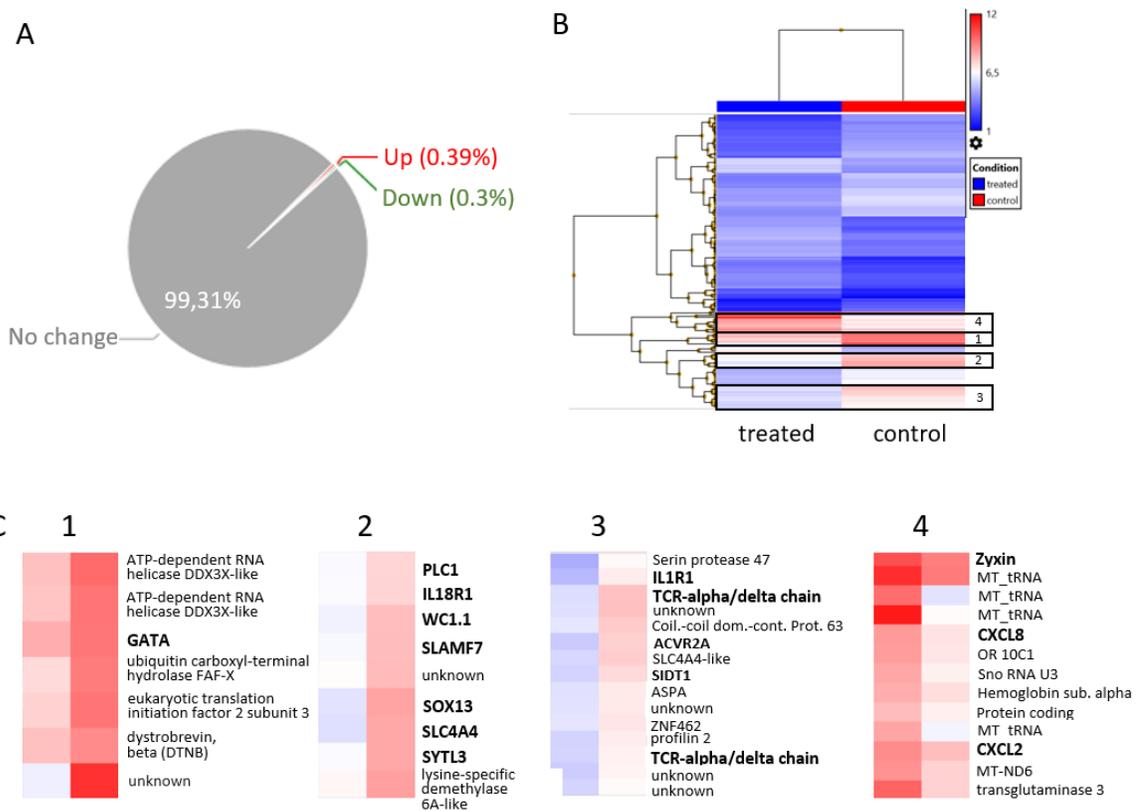
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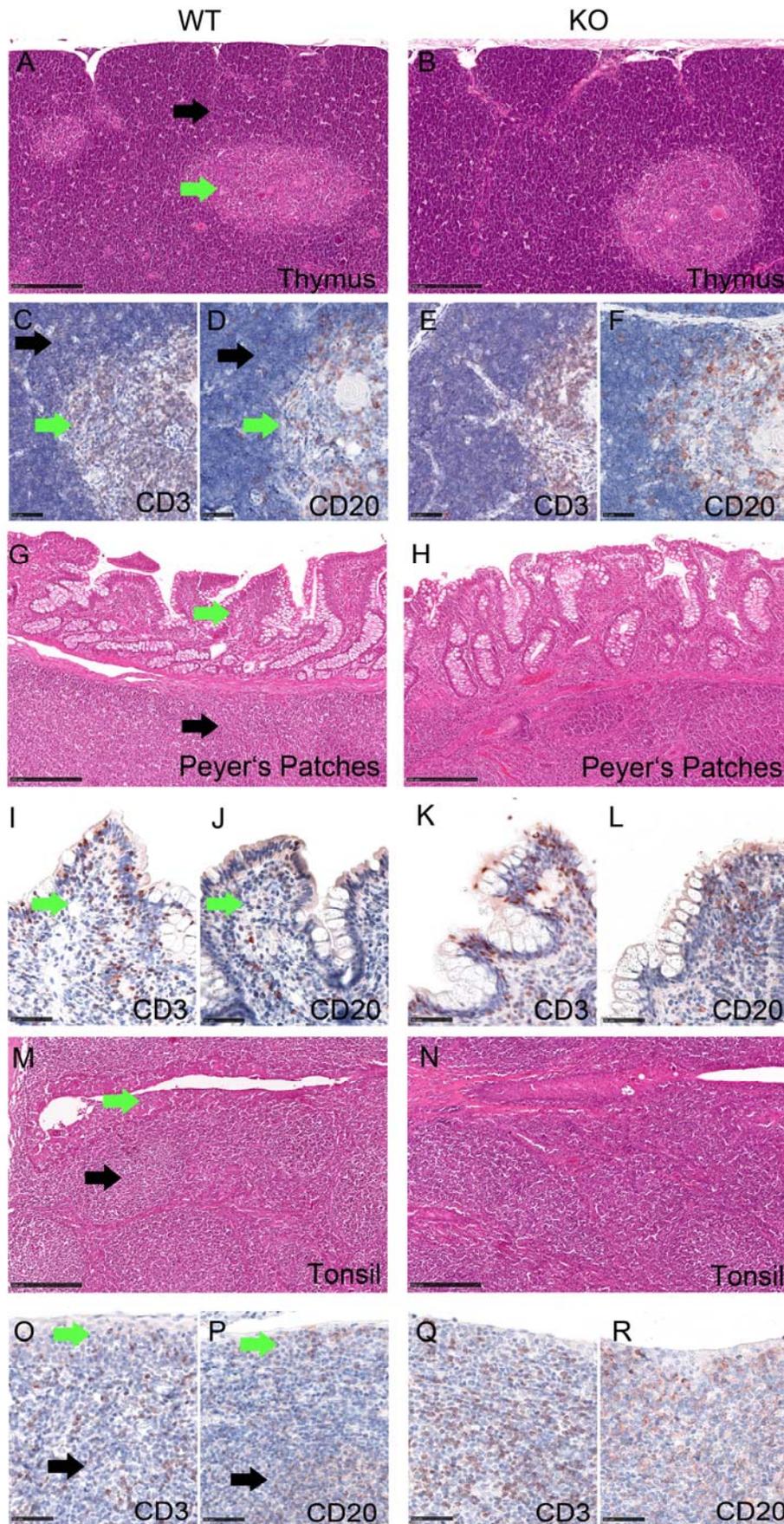
472 **Figure 6: No $\gamma\delta$ T cells are present in blood and spleen of TRDC-Knockout pigs. (A)**
 473 PBMC of TRDC-Knockout and wild type pigs were stained with anti-CD3, anti- δ chain and
 474 anti- $\gamma\delta$ T cell-specific CD3 mABs. CD3 positive cells were gated and δ chain vs. $\gamma\delta$ T cell-
 475 specific CD3 dotblots are displayed. About 20% of peripheral blood T cells were $\gamma\delta$ T cells in
 476 wild type pigs while no $\gamma\delta$ T cells were detected in TRDC-Knockout pigs. **(B)** PBMC and
 477 spleen cells were isolated and stained for CD2 and $\gamma\delta$ T cell-specific CD3. Typically in wild
 478 type pigs the majority (80%) of $\gamma\delta$ T cells in the blood belong to the CD2 negative phenotype
 479 (left dot blot), while in the spleen the majority (66%) of $\gamma\delta$ T cells are CD2 positive (middle
 480 dot blot). No $\gamma\delta$ T cells of either type were detected in the spleen of TRDC-Knockout pigs.
 481 **(C)** Most $\gamma\delta$ T cells are CD4 and CD8 negative. Accordingly TRDC-Knockout pigs have
 482 minimal numbers of CD4/CD8 double negative T cells. **(D)** The percentage of lymphocyte
 483 subpopulations of all lymphocytes without $\gamma\delta$ T cells were compared of wild type and TRDC-
 484 Knockout pigs. No significant difference of any lymphocyte subpopulation was detected.



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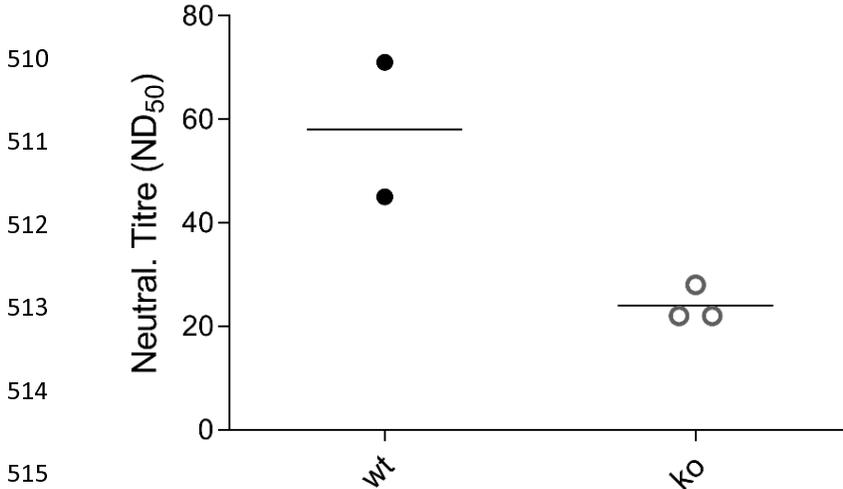
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487 **Figure 7: Transcriptional changes in PBMC of TRDC-Knockout pig refelect the loss of**
488 **$\gamma\delta$ T cells. (A)** Only 0.7 % of genes were regulated more than 2 fold in PBMC of TRDC-
489 Knockout pigs. **(B)** Using hirachical clustering up-regulated and down-regulated genes were
490 sorted acording to the expression levels. **(C)** down-regulated (1,2,3) and upregulated (4)
491 genes from the hirachical clustering (B) were characterized in more detail. (1) all annotated
492 genes of this cluster were expressed preferentially by T cells. GATA3 is highly expressed by
493 $\gamma\delta$ T cells.
494 (2) Cluster 2 contains genes like WC1.1 and SOX13 which are preferentially expressed by $\gamma\delta$
495 T cells to a various degree. (3) T cell receptor genes were found in cluster 3. In addition, IL-
496 1R (3) and IL-18R (2) were downregulated in PBMC of TRDC-Knockout pigs. (4) Genes
497 which were upregulated contain genes which were preferentially expressed by granulocytes
498 and monocytes in human PBMC like Zyxin, CXCL8 and CXCL2.



500 **Figure 8: No differences between wildtype and TRDC-Knockout pigs were detected by**
501 **histological examination of lymphoid tissues.** Lymphoid tissues from wild type (WT: A, C,
502 D, G, I, J, M, O, P) and knockout (KO: B, E, F, H, K, L, N, Q, R) domestic pigs. Thymus (A,
503 B) with cortex (black arrow) and medulla (green arrow), Peyer's Patches (G, H) with mucosa
504 associated lymphoid follicle (black arrow) and absorptive epithelium (green arrow) containing
505 intraepithelial, and transmigrating lymphocytes, Tonsil (M, N) with mucosa associated
506 lymphoid follicle (black arrow) and mucosal epithelium (green arrow) containing intraepithelial
507 and transmigrating lymphocytes. H&E stain (A, B, G, H, M, N), immunohistochemistry (C-F, I-
508 L, O-R), using anti-CD3 or anti-CD20 antibody as indicated, ABC method, AEC chromogen

509



516

517 **Figure 9:** Diagram showing the titers of virus-neutralizing serum antibodies of 2 syngenic wt
518 (wt) and 3 TRDC-Knockout (ko) pigs at day 21 post vaccination, given as the maximal
519 reciprocal dilution of the sera that is able to neutralize 50% of 100 TCID₅₀ infective doses of
520 CSFV C-strain calculated according to Spaerman-Kaerber. The mean values for both groups
521 are shown as horizontal lines. The P value determined by statistical analysis is 0.0428.

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635

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637

638 **Table 1: Results of the intracytoplasmic microinjection of CRISPR/Cas9 targeting exon**
 639 **4 of the porcine TRDC locus in porcine zygotes.**

Recipient	Microinjected plasmids	Number of transferred embryos	Pregnant*	Born piglets	No. of TRDC-KO
#695	pX330-TRDCEX4#1, pX330TRDCEX4#2	35	+	5	2
#696	pX330-TRDCEX4#1, pX330TRDCEX4#2	35	+	9	4
Total: 2		70	2/2 (100%)	14 (eff. 20%)	6/14 (42.9%)

640 *Pregnancy was detected on day 25

641 **Table 2: Results from somatic cell nuclear transfer employing genetically modified**
 642 **cells as donor cells.**

Recipient	Cell clone*	Number of transferred embryos	Pregnant	Born piglets (stillborn)	No. of TRDC-KO of liveborn
#8106	H2	90	+	2 (2)	n.d.
#738	H2	90	+	3 (1)	2/2
#8115	D12	97	+	3 (3)	n.d.
#737	D12	96	+	9 (2)	4/7
Total: 4		373 (avg. 93.3)	4/4 (100%)	17 (cloning efficiency 4.6%)	6/9 (66.7%)

643 * cell clone D12: mixed population of knockout and wild type cells, cell clone H2: pure
 644 knockout cells.

645