Advance Publication

Experimental Animals

1 **Model animals**

2 **Humanized CD36 mouse model supports the preclinical evaluation of**

3 **therapeutic candidates targeting CD36**

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Supplementary Figure: refer to J-STAGE

Abstract

CD36 (also known as scavenger receptor B2) is a multifunctional receptor that mediates lipid uptake, advanced oxidation protein products, and immunological recognition, and has roles in lipid accumulation, apoptosis, as well as in metastatic colonization in cancer. CD36 is involved in tumor immunity, metastatic invasion, and therapy resistance through various molecular mechanisms. Targeting CD36 has emerged as an effective strategy for tumor immunotherapy. In this study, we have successfully generated a novel CD36 humanized mouse strain where the sequences encoding the extracellular domains of the mouse *Cd36* gene were replaced with the corresponding human sequences. The results showed that CD36 humanized mice only expressed human CD36, and the proportion of each lymphocyte was not significantly changed compared with wild-type mice. Furthermore, CD36 monoclonal antibody could significantly inhibit tumor growth after treatment. Therefore, the CD36 humanized mice represent a validated preclinical mouse model for the evaluation of tumor immunotherapy targeting CD36.

Keywords: CD36; Colon cancer; Humanized mouse model; Immunotherapy; Monoclonal antibody

Introduction

The transmembrane protein CD36 is a membrane glycoprotein expressed on the cell surface in multiple cell types, including platelets, mononuclear phagocytes, adipocytes, hepatocytes, myocytes, and some epithelia[1]. Clinical studies have found significant upregulation of CD36 expression in tumor tissues of cervical cancer[2, 3], gastric cancer[4], hepatocellular carcinoma[5], and ovarian cancer[6], which promotes tumor growth, metastatic invasion, and therapy resistance of these tumors through CD36-mediated lipid metabolism.[7].

CD36 expression was significantly higher in primary Colorectal Cancer (CRC) tumor tissue than in normal colonic mucosa, and 5-year survival was lower in CRC patients with high CD36 mRNA expression than in those with low CD36 mRNA expression [8].CD36 is involved in CRC development by promoting proteasome-dependent ubiquitination of Glypcian 4 (GPC4) to inhibit the β-catenin/c-myc axis[9]. The non-coding RNA (lncRNA) TINCR was found to inhibit miR-107 expression and activate CD36, which further inhibited CRC cell proliferation and promoted CRC cell apoptosis[10]. Inhibition of fatty acid synthase (FASN) was found to upregulate CD36 expression in FASN knockout CRC cells and CRC models based on transgenic mice with hetero- and homozygous deletions of FASN, thereby promoting the proliferation of CRC cells[8]. CRC with high metastatic potential expresses higher levels of CD36, which promotes CRC metastasis by upregulating MMP28 and increasing E-calmodulin cleavage[11]. Lipid droplets (LD) are a common feature of cancer cell adaptation to tumour microenvironment (TME) acidosis and a key driver of increased

66　cancer cell aggressiveness[12]. It was found that acidosis of TME induces plasma

67　membrane transport of CD36 via TGF-β2, which promotes LD formation and

68　enhances metastasis and invasion of CRC[13]. Therefore, CD36 is closely associated

69　with CRC development, growth, tumor immunity, and metastatic invasion, suggesting

70　that inhibition of CD36 may be necessary to improve the efficacy of FASN-targeted

71　therapy.

72　　　Humanized mice include immunodeficient mice xenografted with human cells or

73　tissues as well as mice expressing human gene products[14]. Patient-derived

74　xenografts or cell-derived xenografts (PDX/CDX) models based on immune

75　reconstituted mice or target gene humanized mice are the most used for preclinical *in*

76　*vivo* efficacy evaluation of antibodies for tumor immunotherapy. However, immune

77　reconstituted mice have limitations such as species specificity of histocompatibility

78　complex (MHC) antigens, underdevelopment of the immune system, impaired class

79　switching and affinity maturation of immunoglobulins[15]. Therefore, a murine model

80　used to conduct preclinical testing of anti-hCD36 Abs would be an invaluable tool for

81　defining their mechanism of action and potential clinical utility. In this study, we

82　described the generation and characterization of the humanized CD36(hCD36) mouse

83　strain and validation of their use in studying CD36-targeting therapies for potential

84　application in *in-vivo* anti-tumor activity.

85　Materials and methods

86　Reagents and materials

87　　　RP23-115H10 (cloned in the pBACe3.6 vector) and CH17-134L21 (cloned in the

88 pBACGK1.1 vector) were obtained from the BACPAC Resources Center at BACPAC

89 Genomics. ShunRan biology provided MC38 (colon adenocarcinoma) cells, which

90 were cultivated and maintained in Dulbecco's Modified Eagle's medium (DMEM)

91 with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Antibodies against mouse

92 cell-surface molecules including mCD45-BV510 (clone 30-F11), mGr-1- PerCP

93 (clone RB6-8C5), mCD4-BV421 (clone GK1.5), mF4/80-FITC (clone BM8), mCD4-

94 BV510 (clone RM4-5), mCD8a-PE (clone 53-6.7), mFoxp3-APC (clone FJK-16S),

95 mCD19-FITC (clone 6D5), mTCR βchain- PerCP/Cy5.5 (clone H57-597), mCD11c-

96 BV605 (clone N418), mCD11b-PE (clone M1/70), and mCD11b-V450 (clone V450)

97 were purchased from Biolegend(USA). Antibodies against mouse cell-surface

98 molecules including mCD3-V450 (clone 17A2), mNK1.1-PE-Cy7 (clone PK136)，

99 and mTCR βchain- APC (clone H57-597) were purchased from BD

100 Pharmingen(USA). Antibody against mouse cell-surface molecule CD36 Monoclonal

101 antibody-APC (clone HM36) was purchased from eBioscience. Antibody against

102 human cell-surface molecule hCD36-PE (clone 5-271) was purchased from

103 Biolegend(USA). Other reagents including anti-CD16/32, PE Mouse lgG2a, and κ

104 Isotype Ctrl(FC) Antibody (clone MOPC-173) were purchased from Biolegend(USA).

105 Armenian Hamster lgG Isotype Control-APC (clone eBio299Arm) was purchased

106 from Invitrogen.

Animal experiments

108   C57BL/6 mice were purchased from Biocytogen Pharmaceuticals (Jiangsu,

109 China) Co., Ltd. Flp mice were purchased from Institute for Laboratory Animal

110 Resources, NIFDC. CD36 humanized mice (hCD36 mice) were provided by

111 Biocytogen Pharmaceuticals (Beijing, China) Co., Ltd., production license No. SCXK

112 (Su) 2021-0003. This study was carried out in strict accordance with the

113 recommendations in the Guide for the Institutional Animal Care and Use Committees

114 (IACUC) guidance. All animal studies were performed according to the protocol

115 approved by the animal care and use committee of Biocytogen Pharmaceuticals

116 (Approval number: PS-01-2203022). The SPF barrier facility kept the test animals at

117 a constant temperature of (22 ± 2°C), with a 12 h/12 h light/dark cycle, and were free

118 access to food and drink. All surgery was performed under sodium pentobarbital

119 anesthesia, and all efforts were made to minimize suffering. Mice were euthanized

120 with CO_2 to minimize or alleviate the animals' suffering.

Construction of the hCD36 targeting vector

122   On the C57BL/6J genetic background, a partial sequence of mouse exon 4 to a

123 partial sequence of exon 15 of approximately 43 kb was replaced with a partial

124 sequence of exon 3 to a partial sequence of exon 14 of approximately 27 kb

125 containing the human *CD36* (Fig. 1A). Homology areas, human DNA, an Frt-flanked

126 Neo resistance cassette, and a diphtheria toxin A (DTA) cassette were all included in

127 the construction of the hCD36 targeting vector. The vectors pUNS-Neo-2G and pES-

128 Fte were provided by Biocytogen Pharmaceuticals (Beijing, China) Co., Ltd. The

129 mouse and human genomic DNA fragments of A, B, and C were amplified by PCR

130 using DNA derived from Mouse BAC and Human BAC as templates. Primer designs

131 for fragments A, B, and C are shown in Table S1. Intermediate vector 1 (pUNS-Neo-

132 2G-A1A2B1B2) and intermediate vector 2 (pES-FTe-C1-ORI-C2) were obtained by

133 the Gibson assembly method, respectively. Intermediate vector 1 linearized by SmaI

134 was electroporated into the mouse BAC which was modified by intermediate vector 2

135 to obtain intermediate vector 3 (pES-FTe-LR-RR). Finally, the intermediate vector 3

136 linearized by ScaI was electroporated to the human BAC(with Neo) to form the pES-

137 Fe-ABC vector (the hCD36 targeting vector).

Generation of humanized CD36 mice

139   The correct targeting vector was transfected into C57BL/6J embryonic stem (ES)

140 cells (Biocytogen Pharmaceuticals (Beijing) Co., Ltd.) by electroporation. G418-

141 resistant ES clones were assayed using Southern Blot techniques to confirm the

142 integration of the exogenous gene. The ES clone cells which had correct sequencing

143 were injected into BALB/c blastocysts and implanted into pseudo pregnant females to

144 produce F0 chimeric mice. F0 chimeric mice were mated with Flp mice to obtain F1

145 heterozygous mice with the *Neo* allele deleted. Afterwards, F1 heterozygous mice

146 were mated with each other to obtain hCD36 mice (Unless otherwise stated, hCD36

147 mice below refer to homozygous hCD36 mice).

Analysis of the hCD36 targeting vector, the ES cells, and the

F1 mice

150   The hCD36 targeting vectors were subjected to restriction digestion analysis.

151 These vectors were digested with ScaI (Thermo, FD0434), EcoRV (Thermo, FD0304),

152 and NcoI (Thermo, FD0574), respectively. The vectors which conformed by the

153 restriction digestion analysis would be sequenced for confirmation. The G418-

154 resistant ES clones were subjected to Southern Blot assay. We digested cellular DNA

155 with ScaI(NEB, R3122S) or EcoNI(NEB, R0521S) and hybridized using 2 probes,

156 respectively (Restriction enzyme digestion sites are shown in Fig. 1A; primer design

157 of the probes is shown in Table S3). Identification of the DTA cassette in ES positive

158 clones by PCR (The primer sequences were shown in Table S2). The ES clone cells

159 which had correct sequencing were used for blastocyst injection. Somatic cells from

160 female WT and F1 mice were collected for genotype identification by PCR (Primer

161 design for genotype identification of F1 mice is shown in Table S4, and the

162 identification strategy has been Fig. 1A). Genetic sequencing was performed on key

163 positions of F1 mice screened by PCR. The correctly sequenced F1 mice were used

164 for amplification and preparation of hCD36 mice.

165 Analysis of human CD36 mRNA expression and full-length
166 Sanger sequencing of CDS (Coding DNA sequence) in
167 hCD36 mice

168 Lung tissues of hCD36 mice and WT mice (C57BL/6 mice) were harvested for

169 extracting total RNA using RNAprep Pure Cell / Bacteria Kit (TIANGEN, DP430).

170 The mRNA expression of hCD36 and mCD36 was determined using GAPDH as the

171 internal control. Primer annealing temperatures and the number of cycling were set as

172 follows: initial 94 °C for 2 min, followed the first step by 15 cycles of 98 °C for 10 s,

173 67 °C for 30 s, and 68 °C for 30 s, the second step by 25 cycles of 98 °C for 10 s,

174 57 °C for 30 s, and 68 °C for 30 s; an additional extension at 68 °C for 5 min; and

175 finally held at 16 °C. The primer sequences were shown in Table S5. The qualified

176 RNA was used for cDNA library construction. The cDNA obtained by reverse

177 transcription was amplified by RT-PCR. Primer annealing temperatures and the

178 number of cycling were set the same as for the amplification of *CD36* mRNA. Finally,

179 qualified cDNA library was sequenced by the company GENEWIZ.

180 **Protein expression analysis of CD36 and**
181 **immunophenotyping in hCD36 mice**

182 CD36 expression was examined in bone marrow derived from WT mice and

183 heterozygous hCD36 mice, and hCD36 mice (peritoneal exudative macrophages)

184 using anti-mouse CD36 antibody-APC (Biolegend, 102611) and anti-mouse CD36-PE

185 (eBioscience^{TM}, 17-0362-82). And hCD36 was detected with an anti-human CD36

186 antibody (Biolegend, 336205) in peritoneal exudative macrophages. We analyzed the

187 expression of CD36 on immune cells in the blood of WT mice and hCD36 mice using

188 flow cytometry. ACK lysis buffer (Beyotime, China) was first added to the

189 anticoagulated blood to remove red blood cells. These cells were then co-incubated

190 with a mixture of LD-NIR (Biolegend, USA) and anti-mCD16/32 (Biolegend, USA,

191 clone 93) respectively for 10 min at 4°C for deadwood staining and blocking of non-

192 specific binding. Finally, immune cells in these cell suspensions were stained at 4°C.

193 After each staining, cells were washed with PBS to remove unbound labeled

194 antibodies. After staining, multicolor flow cytometry of the cells was performed using

195 Attune NxT (Thermo Fisher Scientific, USA), and analysis of the data was performed

196 using FlowJo 10. We further analyzed the development, differentiation, and

197 distribution of immune cells in the spleen, lymph nodes, and blood of WT mice and

198 hCD36 mice, respectively. The spleen cell suspensions and lymph node cell

199 suspensions were processed and analyzed with the same procedure as anticoagulated

200 blood.

Routine mouse blood test

202   Peripheral blood of female WT and hCD36 mice (n=8, 6-8 weeks old) was

203 collected into EDTA blood collection tubes after anesthesia with sodium pentobarbital.

204 The counts of red blood cells (RBC), white blood cells (WBC), neutrophils (NEUT#),

205 lymphocytes (LYMPH#), monocytes (MONO#), hemoglobin (HGB), and platelets

206 (PLT) in the peripheral blood of mice were determined using a fully automated

207 modular blood fluid analyzer (Sysmes, XN-1000); the pressure of red blood cells

208 (HCT), the mean red blood cell volume (MCV) and red blood cell distribution width

209 (RDW); mean platelet volume (MPV), hemoglobin content (MCH) and mean

210 hemoglobin concentration (MCHC).

Biochemical examination of mouse peripheral blood

212   Peripheral blood of female WT and hCD36 mice (n=8, 6-8 weeks old) was

213 collected into heparin collection tubes after anesthesia with sodium pentobarbital, and

214 the supernatant was collected after centrifugation. The concentrations of alanine

215 aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

216 (ALP), albumin (ALB), glucose (GLU), urea (UREA), creatinine (CREA), total

217 cholesterol (TC), triglycerides (TG), total protein (TP), alkaline phosphatase (ALP),

218 serum amylase (AMY), phosphorus (P), creatine kinase (CK), high-density

219 lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) in the peripheral blood of

220 mice were determined using a fully automatic biochemical analyzer (Hitachi, 3110).

In vivo efficacy evaluation of CD36 monoclonal antibody

222   Six- to eight-week-old humanized female hCD36 mice were housed in the

223 specific-pathogen-free (SPF) barrier facility of the Animal Center of Biocytogen

224 Pharmaceuticals (Beijing) Co., Ltd. in the individually ventilated cage. The

225 experimental animals were acclimatized for 7 days before being used in experiments.

226 5E5 Murine colon cancer MC38 cells were subcutaneously implanted into hCD36

227 mice on the right dorsal side in a volume of 0.1ml per mouse. The tumor volume was

228 measured once a day from day 0 after inoculation, and the tumor volume was

229 calculated by the formula: $0.5 \times$ long diameter $\times$ short diameter^2. Mice were randomly

230 grouped as tumors reached an average of 100 mm^3. Then mice were treated with PBS,

231 anti-CD36 chimeric human-mouse monoclonal antibody (subsequently abbreviated as

232 1G04 which was made in house.) through i.p. injection. The antibody was

233 administered three times a week for six consecutive doses. Animal well-being and

234 behaviors were monitored once a day during the experiment process. We measured

235 the tumor volume and weight of the animals twice weekly. The animals were

236 euthanized at the end of the experiment, and the relative tumor growth inhibition (TGI)

237 rate was measured. The antitumor efficacy is expressed as tumor growth inhibition in

238 terms of tumor volume (TGI$_{TV}$). The TGI$_{TV}$ in percent was calculated as below:

239 $TGI_{TV}(\%) = [1 − (T_i − T_0)/(C_i − C_0)] \times 100$; Where T_i = mean tumor volume of the

240 drug-treated group on the final day of the study, T_0= mean tumor volume of the drug-

241 treated group on the first dosing day, C_i = mean tumor volume of the control group on

242   the final day of the study, C_0= mean tumor volume of the control group on the first

243   dosing day.

Statistical analyses

245   Mean±SEM was used to assess the results. Statistical analysis was performed

246   using SPSS 19 and graphical plotting of data was performed using Graph Pad Prism 7

247   software. Student's t-test and one-way analysis of variance (ANOVA) were used for

248   the comparison of all data. Statistical significance was required to meet a *P* value <

249   0.05. * *p* <0.05, ** *p* < 0.01, *** *p* < 0.001, and **** *p* < 0.0001.

Results

Generation of humanized CD36 heterozygous mouse model

252   The fragment size after ScaI digestion should be 30,955 bp, 8094 bp, 5618 bp,

253   2801 bp, 856 bp, and 96 bp. The fragment size after EcoRV digestion should be

254   40,001 bp, 4020 bp, 2203 bp, 1427 bp, 540 bp, and 229 bp. The fragment size after

255   NcoI digestion should be 22015 bp, 10334 bp, 5337 bp, 3259 bp, 2678 bp, 1841 bp,

256   910 bp, 758 bp, 571 bp, 378 bp, and 339 bp. As shown in Fig. 1B, the hCD36

257   targeting vector No. 17 met the requirements of restriction digestion analysis and was

258   then sequenced to confirm (data not shown). Southern Blot was performed on G418-

259   resistant ES clones and the results showed that No. 1D02, No. 1D06, No. 2B04, No.

260   2D02, No. 2D03, No. 3F05, No. 1D08, and No. 2E06 were positively confirmed (Fig.

261   1C). Sequenced of these positive clones showed that the four positive clones No.

262   2B04, No. 1D08, No. 2D03, and No. 3F05 were free of random insertions (data not

263   shown). Finally, the correct ES-positive clones were injected into BALB/c blastocysts

264 and then implanted into pseudo pregnant females to produce F0 chimeric mice. F0

265 chimeric mice were mated with Flp mice to obtain F1 heterozygous mice with the

266 Neo allele deleted. Genotype identification of F1 mice somatic cells by PCR showed

267 that the two mice No. F1-01 and No. F1-02 were both positive heterozygous mice

268 (Fig. 1D). The maternal ES positive of mice No. F1-01 and F1-02 was No. 2D03, and

269 PCR identified No. 2D03 without the DTA cassette (S1 Fig). In addition, one of the

270 F1 mice which was sequenced at the critical position showed no random insertion (S4

271 Fig). The F1 mice No. F1-01 and No. F1-02 were used for the amplification and

272 preparation of hCD36 mice.

Generation of hCD36 mouse model

274 The hCD36 mice established based on the C57BL/6 mouse background were

275 obtained by replacing exons 4 to part of 15 of the extracellular domains of CD36

276 encoding the mouse with human exons 3 to 14 (Fig. 1A). The hCD36 mice were

277 generated by mating F1 mice with each other. The full-length Sanger sequencing data

278 of the CD36 CDS in hCD36 mice showed that the sequence of exon 4 to exon 15 of

279 the mouse CD36 gene was correctly replaced with the sequence of exon 3 to exon 14

280 of the human CD36 gene in hCD36 mice (S5 Fig). We assumed that homozygous

281 hCD36 mice only express hCD36, while heterozygous hCD36 mice express both

282 hCD36 and mCD36. We detected CD36 expression in the bone marrow of

283 heterozygous hCD36 mice and found that it expressed both mCD36 and hCD36,

284 while WT mice only expressed mCD36 (results not shown). We analyzed the

285 expression of CD36 mRNA in the lung tissue of hCD36 mice by RT-PCR. The results

revealed that only hCD36 mRNA was detected in hCD36 mice compared to WT mice (Fig. 2A). We then examined hCD36 protein expression in peritoneal exudative macrophages of hCD36 mice and found that only mCD36 was detected in WT mice, while only hCD36 was detected in hCD36 mice (Fig. 2B).

We further evaluated the expression pattern of hCD36 on different immune populations in the humanized mice and compared it with the expression of hCD36 protein in humans. Similar to the pattern found on human immune cells[16-20], hCD36 protein is expressed on macrophages, monocytes, granulocytes, DCs, NK Cells, B cells, CD8+ T cells, CD4+ T cells, and Tregs of hCD36 mice (Fig. 2C). The regulatory region of the CD36 gene was not replaced in hCD36 mice, and the expression of CD36 protein on peripheral blood immune cells of hCD36 mice should be consistent with that of WT mice. However, our results showed that hCD36 protein expression was elevated on macrophages, monocytes, granulocytes, DCs, NK cells, B cells, CD8+ T cells, CD4+ T cells, and Tregs in hCD36 mice compared to mCD36 protein expression in WT mice (Fig. 2C). Actually, it has been shown that introns can enhance transcript levels in eukaryotes by elevating mRNA accumulation and affecting transcription speed, nuclear export, and transcript stability[21]. Transgenic mice carrying the human histone H4 promoter had more extensive tissue expression in response to stimulation with the mixed introns compared to controls[22]. Tissue-specific expression of the mouse CD21 gene is closely associated with the 5'1.6kb region within intron 1 of the CD21 gene[23]. In addition, introns that can be efficiently spliced may make very different contributions in the context of different

308   cells, different promoters, or different coding sequences[21]. It is therefore reasonable

309   to suspect that the partial sequence of exon 3 to exon 14 of the human CD36 gene

310   may contain introns that enhance the expression of hCD36 protein in hCD36 mice.

311   Altogether, our data demonstrate that the hCD36 mice recapitulate the expression

312   pattern of the human CD36 on peripheral blood mononuclear cells (PBMCs),

313   supporting the use of this mouse to study human CD36 as the therapeutic target for

314   immune therapy.

315 Analysis of leukocyte subpopulations and T cell
316 subpopulations

317   To further investigate whether the humanization of CD36 could affect the

318   immune system of mice, we next analyzed the leukocyte subpopulation in the spleen

319   of hCD36 mice by flow cytometry. As shown in Figs 3A and B, the development,

320   differentiation, and distribution of leukocyte subpopulations such as T cells, B cells,

321   natural killer cells (NK), monocytes, dendritic cells (DC), and mononuclear

322   macrophages in the spleen of hCD36 mice were not statistically different from WT

323   mice ($p > 0.05$). T cell subpopulations such as CD4+ T cells, CD8+ T cells, and Treg

324   cells in the spleen of hCD36 mice were also similar to WT mice ($p > 0.05$) (Figs 3C

325   and D). In addition, we further analyzed the development, differentiation, and

326   distribution of leukocyte subpopulations and T cell subpopulations in the blood and

327   lymph nodes of hCD36 mice. We found that leukocyte subpopulations and T cell

328   subpopulations of blood and lymph nodes were not significantly changed as compared

329   with WT mice ($p > 0.05$). (S2 and S3 Figs). Collectively, these results indicate that the

330 development, differentiation, and distribution of immune cells in hCD36 mice are not

331 impaired, and hCD36 mice possess normal immune functions.

Analysis of routine blood and blood biochemistry

333 CD36 expression in non-immune cells mainly includes platelets, immature

334 erythrocytes, podocytes, skeletal muscle cells, adipocytes, and cardiomyocytes[24].

335 We next examined whether blood cell composition and morphology were affected by

336 CD36 humanization. As shown in Fig. 4A, hCD36 mice were measured similarly to

337 WT mice, indicating that humanization does not alter hematocrit composition and

338 morphology.

339 Notably, cardiomyocyte-specific CD36 knockout mice exhibited a significant

340 reduction in cardiac FA uptake and intramyocardial TG content[25]. Hepatocyte-

341 specific CD36 knockout mice exhibited high-fat diet-induced hepatic steatosis and

342 diminished insulin resistance, and blood biochemical assays suggested a progressive

343 decrease in AST and ALT[26]. CD36 also contributes to the progression of chronic

344 kidney disease by mediating renal lipid deposition, lipid peroxidation, and

345 endocytosis of multiple substances by renal cells[27]. Endothelial cell-specific CD36

346 knockout mice also exhibit elevated triglyceride levels, reduced total cholesterol, and

347 increased glucose clearance[28]. These studies suggest that CD36 is closely

348 associated with the normal function and disease development of organs such as the

349 heart, liver, and kidney in mice. We next evaluated the effect of CD36 humanization

350 on the normal function of these organs in hCD36 mice. There was no difference in

351 biochemistry parameters between hCD36 mice and WT mice, indicating that

352 humanization does not alter the health of organs such as the heart, liver, and kidney

353 (Fig. 4B).

In vivo efficacy evaluation of CD36 monoclonal antibody

355 Currently, there are no antibodies that specially recognize hCD36 other than the

356 1G04 (patent WO2021176424A1) which cross-recognizes the mCD36 and hCD36.

357 1G04 is currently in the biological testing phase. This antibody is an anti-CD36

358 human-mouse chimeric monoclonal antibody that was obtained by replacing the Fc

359 region of the mouse antibody ONA-0-V1 with the Fc region of the human antibody

360 lgG1[29]. The Fc region of 1G04 contains L234A/L235A (LALA) modification. The

361 results of the proprietary ELISA assay showed that 1G04 exhibited similar affinity

362 and binding properties for hCD36 and mCD36[29]. 1G04 can inhibit tumor growth by

363 blocking the CD36-mediated lipid uptake of tumor cells[29]. To assess the cross-

364 reactivity of 1G04, we examined its binding to mCD36/hCD36 *in vitro* by flow

365 cytometry, and we confirmed that the 1G04 demonstrated comparable binding to both

366 mCD36 and hCD36 (Fig. 5B). It was reported that 1G04 exhibited significant tumor

367 inhibition in either C57BL/6 mice inoculated with MC38 cells or BALB/c nude mice

368 inoculated with HCT-116 cells[29]. To demonstrate that the enhancement of anti-

369 tumor immunity could be modulated by therapeutic blockade of CD36, hCD36 mice

370 with established WT MC38 tumors were treated with 1G04 at 3mg/kg or 10mg/kg.

371 Compared with the control group, 1G04 treatments at the dosage of 10mg/kg and

372 3mg/ kg achieved significant TGI at 47.3% and 31.6%, respectively (Fig. 5C). And

373 neither the low-dose group nor the high-dose group induced significant body weight

374   change in the experimental animals compared with the control group ($p > 0.05$)(Fig.

375   5D). Therefore, we successfully validated the inhibitory effect of 1G04 on colon

376   cancer tumor growth in hCD36 mice. The hCD36 mice could be used for preclinical

377   *in vivo* efficacy evaluation of anti-hCD36 antibody.

378   Discussion

379   CD36 is actively involved in the growth, tumor immunity, metastatic invasion,

380   and drug resistance of various tumors through lipid metabolism[30]. Blocking CD36-

381   mediated lipid metabolism is a strategy that can be considered for future tumor

382   therapy. However, a lack of mouse models that faithfully recapitulate the human

383   expression patterns of hCD36 has hindered progress in evaluating the anti-tumor

384   effect of hCD36-targeting antibodies *in vivo*. Here, we developed a hCD36 mouse

385   model by replacing the extracellular region of mCD36 with the human counterpart,

386   which allowed for testing the therapeutic potential of anti-hCD36-targeting antibody.

387   Expression of hCD36 was only detectable in hCD36 mice while mCD36 was not

388   expressed. Next, the distribution of lymphocyte subpopulations in the blood, lymph

389   nodes, and spleen of the hCD36 mice was consistent with that of WT mice, indicating

390   that humanized mice did not affect the distribution of lymphocyte subpopulations.

391   Furthermore, the immune function, blood cell composition and morphology, and vital

392   organ function were not affected by humanization.

393   We further validated the inhibitory effect of 1G04 on colon cancer tumors in

394   hCD36 mice. Our data showed that targeting the hCD36 with blocking antibody was a

395   promising strategy to enhance antitumor immunity and reduce tumor burden.

However, most of the development of CD36 antibodies is in the preclinical and biological testing stage, and there are few CD36 antibodies whose clinical indications are used for tumor immunotherapy. Although it is a limitation of validation of the hCD36 mice due to lacking of specially recognizing human CD36 monoclonal antibodies, this implies that there is much deeper research on CD36 to develop human-specific CD36 antibodies. In addition, with the emergence of drugs utilized for metabolic inflammatory syndrome and the cardiovascular class of targeting CD36 antibodies in the future, hCD36 mice could also be able to further accelerate the development of this field of antibodies from preclinical to clinical progress. In fact, the use of humanized mice in human metabolic inflammatory syndrome and cardiovascular disease research includes the exploration of mechanisms of action, preclinical pharmacodynamic and toxicological evaluation, and target discovery[14, 31-33]. Therefore, hCD36 mice could also further discover the potential association of CD36 with different targets in several diseases, thus facilitating drug development for these diseases.

Conclusion

We have successfully generated hCD36 mice by a genetic engineering approach and validated the inhibitory effect of 1G04 on colon cancer tumor growth in these mice. The hCD36 mice should be a valuable preclinical mouse model for the evaluation of tumor immunotherapy targeting CD36.

Authorship contributions

Xiulong Xie: Validation, Formal analysis, Writing - Original Draft. Zhenlan Niu:

418   Resources, Analysis and interpretation of data, Writing- Reviewing and Editing.

419   Linlin Wang: Conception and design of the study, Acquisition of data, Analysis and

420   interpretation of data. Xiaofei Zhou: Project administration. Xingyan Yu:

421   Investigation. Hongyan Jing: Visualization. Yi Yang: Funding acquisition, Writing-

422   Reviewing and Editing.

Sources of Founding

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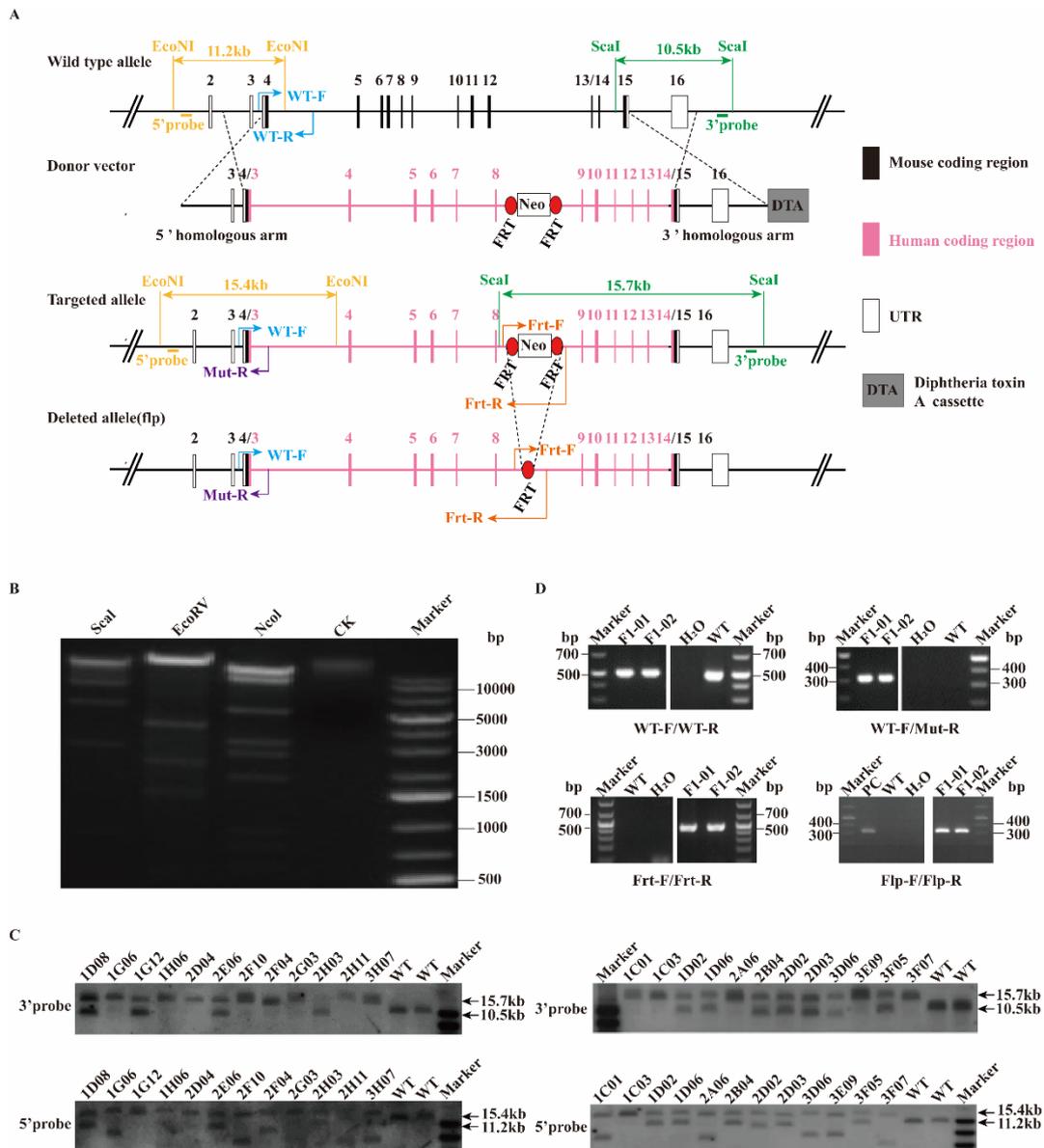
Fig 1. Characterization of the humanized CD36 heterozygous mouse model. A: Generation of the hCD36 mouse strain; B: Restriction digestion analysis of the hCD36 targeting vector No. 17; C: Southern Blot analysis of ES positive clones; D: PCR identification of the genotypes of F1 mice.
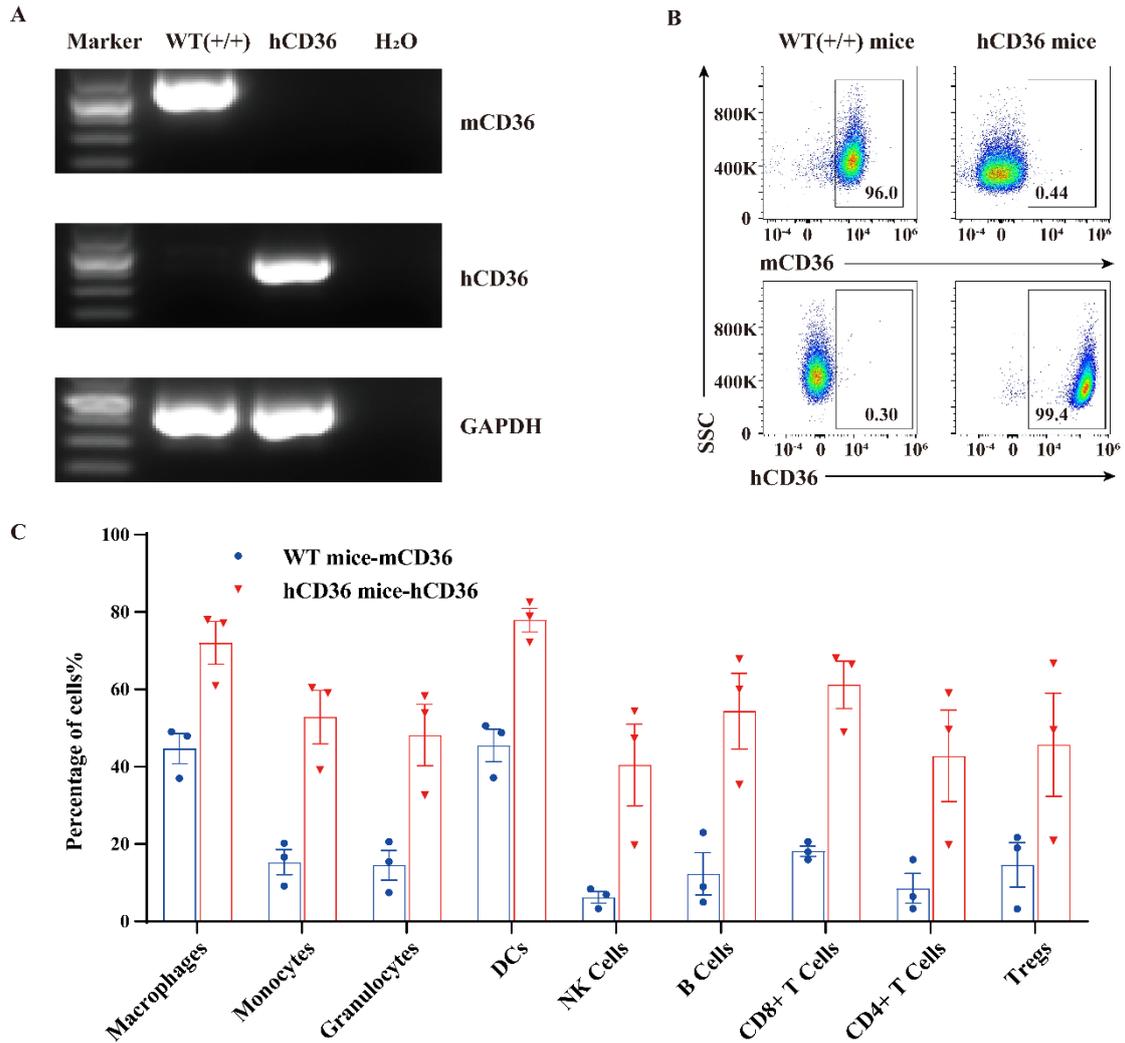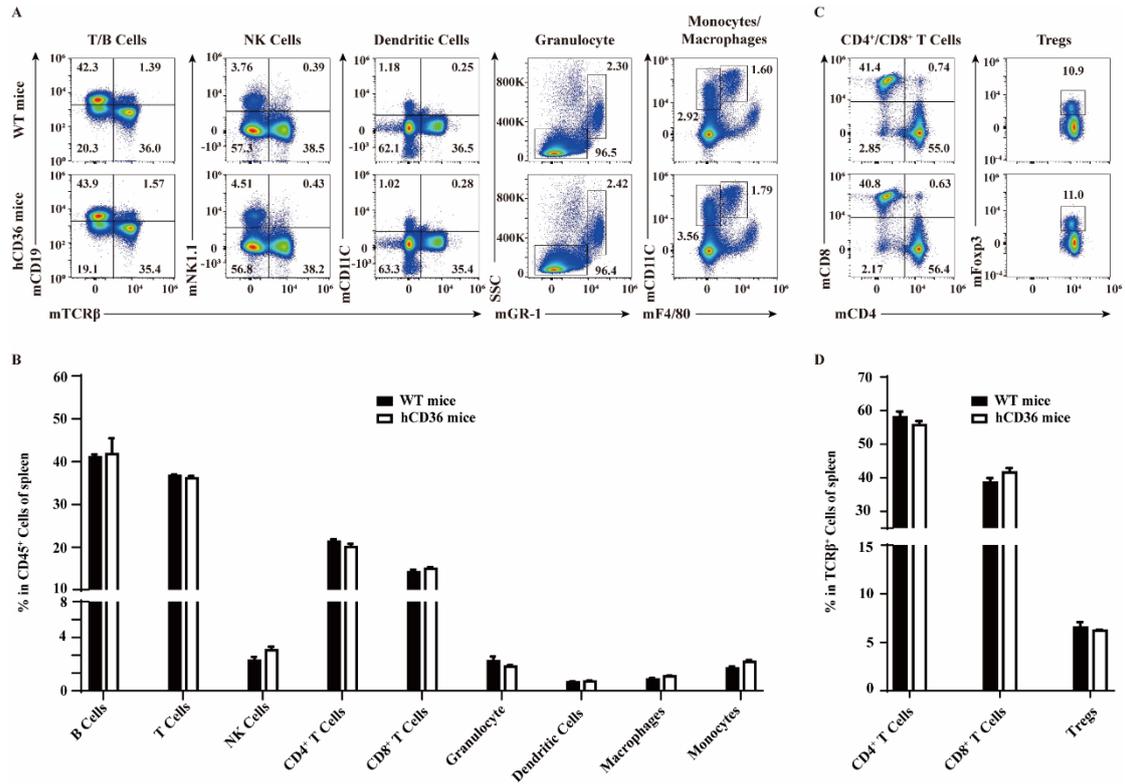
**Fig 2. Characterization of the hCD36 mouse model.** A: Expression of CD36 mRNA in hCD36 mice; B: Expression of CD36 on peritoneal exudative macrophages in hCD36 mice (n=1,7-week-old) ; C: Expression of CD36 on immune cells in the blood of WT mice and hCD36 mice(n=3,8-week-old).

Fig 3. Analysis of immune cells subpopulation in the spleen of hCD36 and WT mice(n=3,8-week-old). A, B: The frequency of T cells, B cells, NK cells, Monocyte, DC cell, and macrophage cells in mCD45 cells. C, D: The frequency of CD4+ T cells, CD8+ T cells, and Treg cells in mCD3 cells.
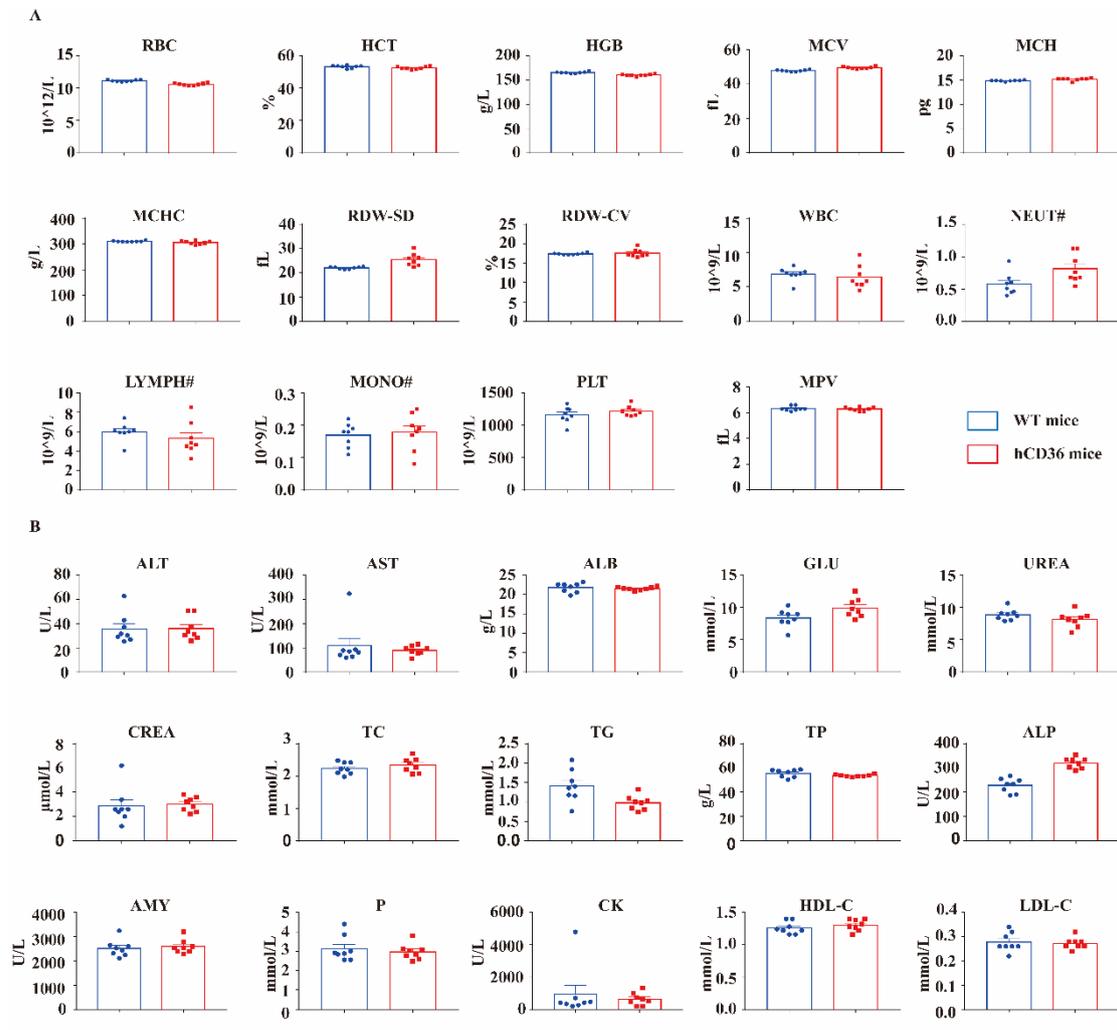
Fig 4. Analysis of complete blood count and blood biochemical in hCD36 and WT mice (n=8,6~8-week-old). A: Analysis of complete blood count in mice; B: Analysis of blood biochemistry in mice.
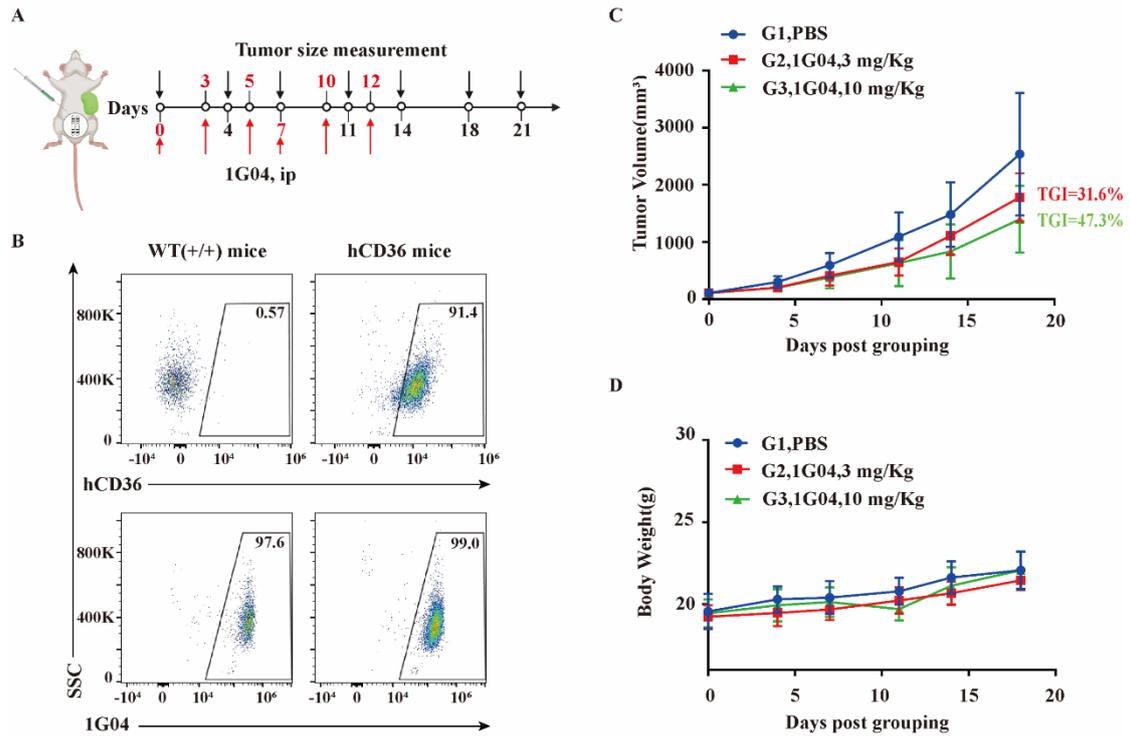
Fig 5. Validation of the efficacy of 1G04 in hCD36 mice (n=6,6~8-week-old). A: Design of *in vivo* drug efficacy programs; B: Analysis of the *in vitro* binding of 1G04 to CD36. C: Tumor volume measurement during the treatment. D: Body weight record during the treatment.
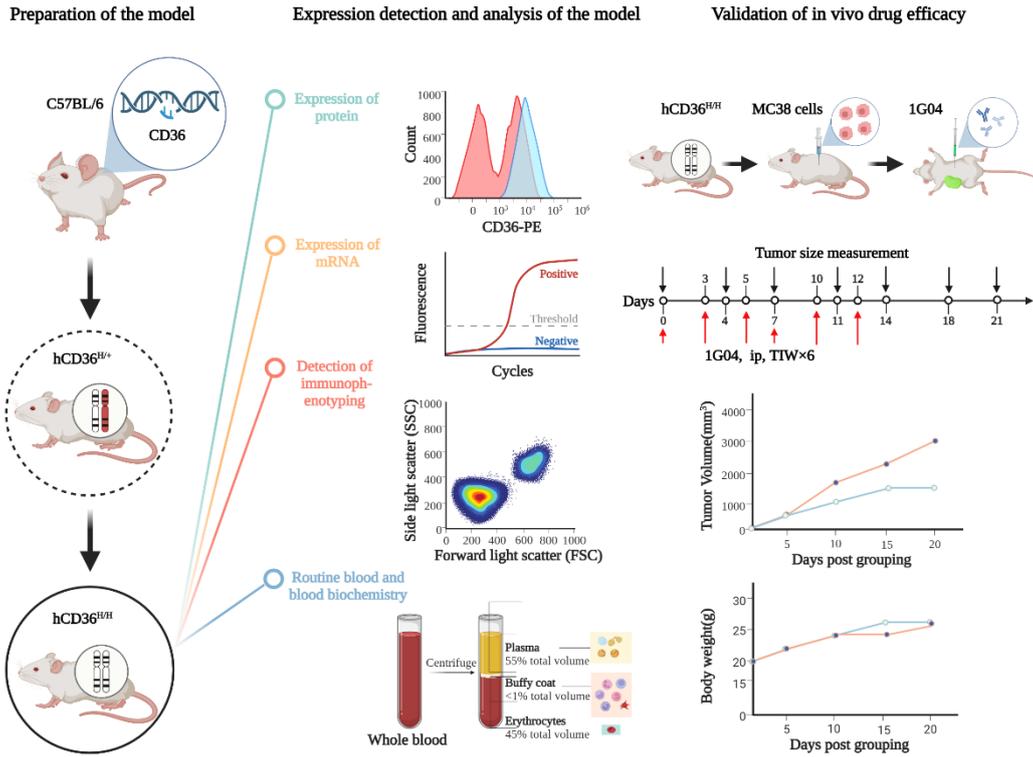
Fig 6. Graphical abstract.

547 **Supplementary data**

548 The following are the Supplementary data to this article:

549 **Table S1. Primer designs for fragments A, B, and C**

Primer	Sequence (5'-3')	Product size(bp)	Template
A1-F	CCTAAGAATACTCTACGGTCACATACCCCCCGGG TGGAGAAACTAGATAGGGCGTG	593	Mouse
A1-R	GATAAGCAGGTCTCCGACTGGCATGAGAATGCCT CCAAAC		BAC
A2-F	ATTCTCATGCCAGTCGGAGACCTGCTTATCCAGA AGAC	342	Human
A2-R	TCCGATTCGCCAGATGATAAGGAACAGTACTAAC ATGCATACCTGTAGACAGC		BAC
B1-F	AACTCCGAGCCCTTCCACCAGTACTCTATCTGGC ACTTAATTGCC	277	Human
B1-R	CCATGCCAAGGAGGTTTATTTTTCCAGTTACTTG AC		BAC
B2-1-F	CTGGAAAAATAAACCTCCTTGGCATGGTAGAGAT GGCCT	735	Mouse
B2-1-R	CTAATATATAAAAAGGCGTGTACAATTTTGCCAA AACTC		BAC
B2-2-F	TTGTACACGCCTTTTTATATATTAGTGACCACTGT G	590	Mouse
B2-2-R	GAAATGTCAGAGCCAGCGTCTTGCCCGGGTGAC ACAAATGATCCAGAAC		BAC
C1-F	AATTTCACACAGGAGGCGCGCCTCGAGCAACTG AATCCTTACCATCTTCTGCC	322	Mouse
C1-R	GAGGGCTTACCGCTTACTAGTCACGTGTCTACAT CCCAGCCACATCACA		BAC
C2-F	GGTTTCCTTGTCGTAGATCTCACGTGTTCTATCTG AGATTCTGTGTCC	542	Mouse
C2-R	GTAAACTCCTCTTCAGACCTGGCGGCCGCCCAC ATGCAGTGCAGAAGGGTGCA		BAC

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551

552 **Table S2. Primer design for identification of DTA cassette in ES positive clones**

Primer	Sequence (5'-3')	Product size(bp)
DTA-F1	CTGGTACACAAGGAAATTATGACGATG	515
DTA-R1	GGTAGTTTGTCCAATTATGTCACACC	
DTA-F2	GAGGCGTGGTCAAAGTGACGTATCC	344
DTA-R2	CCTGACACGATTTCCTGCACAGGC	

553

554 **Table S3. Primer design for Southern Blot confirmation of homologous**

555 **recombination in ES cells**

Probe	Sequence (5'-3')
5'Probe-F	5'-AGATAACAGGCTGGCCTGGAGAAAA -3'
5'Probe-R	5'-GTTGCCCAATACCATAATTTGGAAAAGT-3'
3'Probe-F	5'-CTTTAGCACCAGGTGTGCGTCAGTA-3'
3'Probe-R	5'-GGTCATGCACCAACATCCACACAAC-3'

556

557 **Table S4. Primer design for genotype identification of F1 mice**

Primer	Sequence (5'-3')	Product size(bp)
WT-F	AACCAGTGCTCTCCCTTGATTCTG	WT: 504
WT-R	AAAGCATGCCAGTCTACTCCAGA	
WT-F	AACCAGTGCTCTCCCTTGATTCTG	Mut: 315
Mut-R	GTCGCATCATATAGAGTTGCAGTGG	
Frt-F	TTGCCATGGATCATAGCTGTAGC	Mut-Flp: 490
Frt-R	AGTTACATATGGCTGGCGGCTGCT	(Deleted Neo)
Flp-F2	GACAAGCGTTAGTAGGCACATATAC	Mut: 325
Flp-R2	GCTCCAATTTCCCACAACATTAGT	

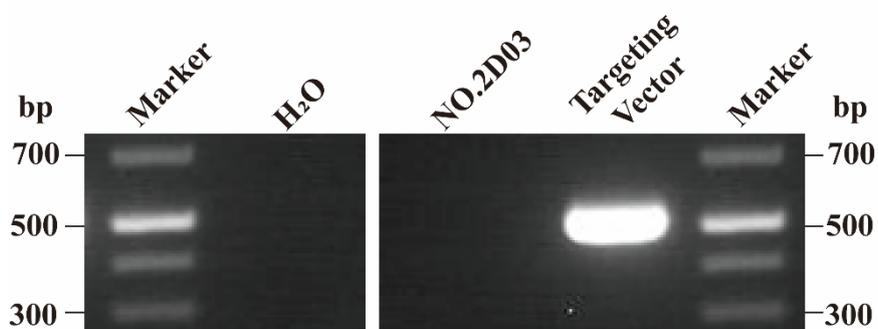**558**

559

Table S5. Primer design for CD36 mRNA expression analysis in hCD36 mice

Primer	Sequence (5'-3')	Product size(bp)
mCD36-F	AAACCCAGATGACGTGGCAA	WT: 634
mCD36-R	TTCAGATCCGAACACAGCGT	
hCD36-F	AATGTAACCCAGGACGCTGA	Mut: 463
hCD36-R	GTGGAAATGAGGCTGCATCTG	
GAPDH-F	TCACCATCTTCCAGGAGCGAGA	WT: 479
GAPDH-R	GAAGGCCATGCCAGTGAGCTT	

561

562

S1 Fig. PCR identification of DTA cassette in targeting vectors and ES-positive

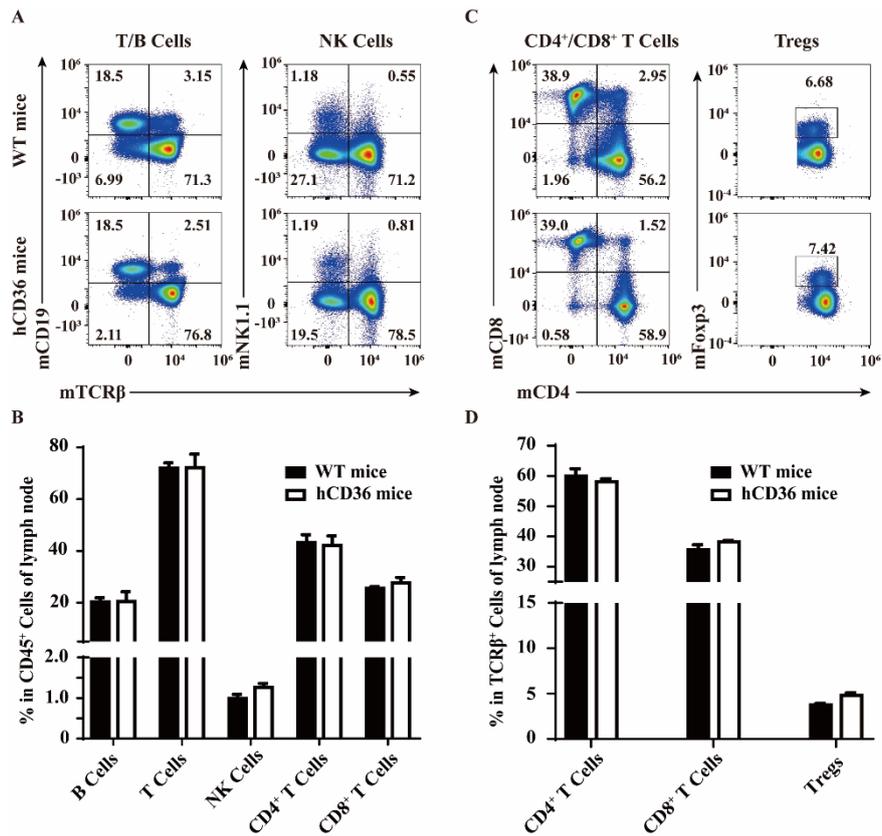**clones No. 2D03.**

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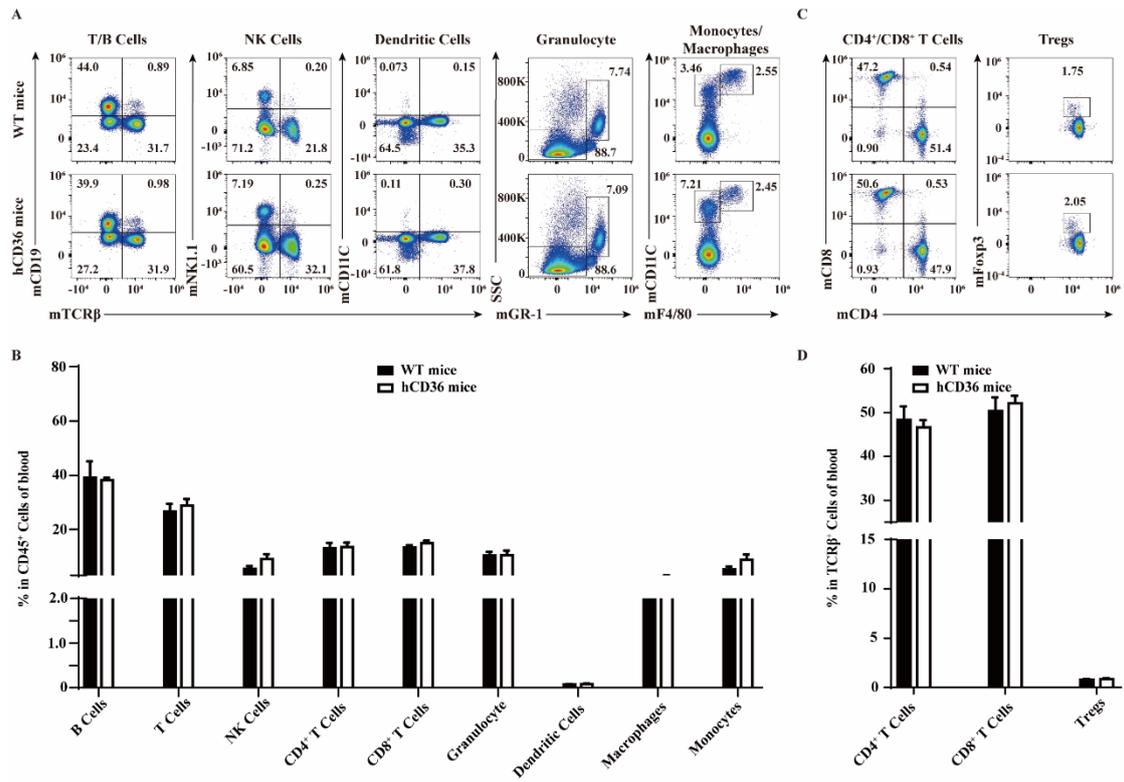
568 **S2 Fig. Analysis of immune cells subpopulation in the blood of hCD36 and WT**

569 **mice(n=3,8-week-old).** A, B: The frequency of T cells, B cells, NK cells, Monocyte,

570 DC cell, and macrophage cells in mCD45 cells. C, D: The frequency of CD4+ T cells,

571 CD8+ T cells, and Treg cells in mCD3 cells.

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574

S3 Fig. Analysis of immune cells subpopulation in the lymph nodes of hCD36 and WT mice(n=3,8-week-old). A, B: The frequency of T cells, B cells, NK cells in mCD45 cells. C, D: The frequency of CD4+ T cells, CD8+ T cells, and Treg cells in mCD3 cells.
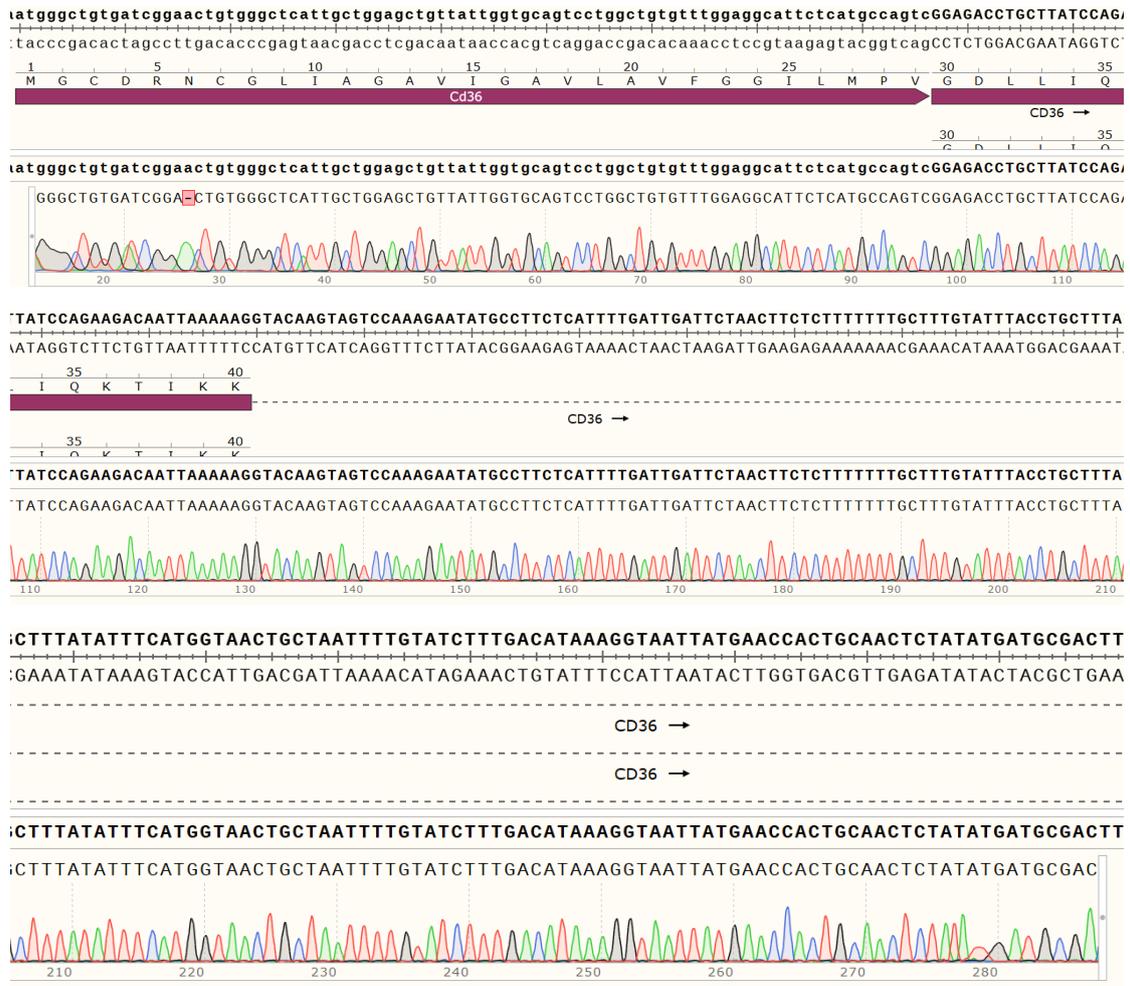
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591 **S4 Fig. One of the F1 mice which was sequenced at the critical position showed**

592 **no random insertion.**

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aatgggctgtgatcggaactgtgggctcattgctggagctgttattggtgcagtcctggctgtgtgtttggaggcattctcatgccagtcggagacctgcttatccagaa
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1　　　5　　　10　　　15　　　20　　　25　　　30　　　35
Met Gly Cys Asp Arg Asn Cys Gly Leu Ile Ala Gly Ala Val Ile Gly Ala Val Leu Ala Val Phe Gly Gly Ile Leu Met Pro Val Gly Asp Leu Leu Ile Gln Ly
Cd36 →

30　　　35
Gly Asp Leu Leu Ile Gln Ly
CD36 →

aatgggctgtgatcggaactgtgggctcattgctggagctgttattggtgcagtcctggctgtgtgtttggaggcattctcatgccagtcggagacctgcttatccagaa
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601

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35　　　40　　　45　　　50　　　55　　　60　　　65　　　70
Gln Lys Thr Ile Lys Lys Gln Val Val Leu Glu Glu Gly Thr Ile Ala Phe Lys Asn Trp Val Lys Thr Gly Thr Glu Val Tyr Arg Gln Phe Trp Ile Phe Asp Val
Cd36 →

35　　　40　　　45　　　50　　　55　　　60　　　65　　　70
Gln Lys Thr Ile Lys Lys Gln Val Val Leu Glu Glu Gly Thr Ile Ala Phe Lys Asn Trp Val Lys Thr Gly Thr Glu Val Tyr Arg Gln Phe Trp Ile Phe Asp Val

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602

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70　　　75　　　80　　　85　　　90　　　95　　　100　　　105
Val Gln Asn Pro Gln Glu Val Met Met Asn Ser Ser Asn Ile Gln Val Lys Gln Arg Gly Pro Tyr Thr Tyr Arg Val Arg Phe Leu Ala Lys Glu Asn Val Thr Gln
Cd36 →

70　　　75　　　80　　　85　　　90　　　95　　　100　　　105
Val Gln Asn Pro Gln Glu Val Met Met Asn Ser Ser Asn Ile Gln Val Lys Gln Arg Gly Pro Tyr Thr Tyr Arg Val Arg Phe Leu Ala Lys Glu Asn Val Thr Gln

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603

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105　　　110　　　115　　　120　　　125　　　130　　　135　　　140
Gln Asp Ala Glu Asp Asn Thr Val Ser Phe Leu Gln Pro Asn Gly Ala Ile Phe Glu Pro Ser Leu Ser Val Gly Thr Glu Ala Asp Asn Phe Thr Val Leu Asn Leu
Cd36 →

105　　　110　　　115　　　120　　　125　　　130　　　135　　　140
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604

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gaccgacaccgtcgacgtagggtatagatagttttagttaaacaagtttactaggagttaagtgaataattgttcagttttagaagatacaaggttcagtcttgaaac

140　　　145　　　150　　　155　　　160　　　165　　　170　　　175
Leu Ala Val Ala Ala Ala Ser His Ile Tyr Gln Asn Gln Phe Val Gln Met Ile Leu Asn Ser Leu Ile Asn Lys Ser Lys Ser Ser Met Phe Gln Val Arg Thr Leu
Cd36 →

140　　　145　　　150　　　155　　　160　　　165　　　170　　　175
Leu Ala Val Ala Ala Ala Ser His Ile Tyr Gln Asn Gln Phe Val Gln Met Ile Leu Asn Ser Leu Ile Asn Lys Ser Lys Ser Ser Met Phe Gln Val Arg Thr Leu

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605

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175　　　180　　　185　　　190　　　195　　　200　　　205　　　210
Leu Arg Glu Leu Leu Trp Gly Tyr Arg Asp Pro Phe Leu Ser Leu Val Pro Tyr Pro Val Thr Thr Thr Val Gly Leu Phe Tyr Pro Tyr Asn Asn Thr Ala Asp Gly
Cd36 →

175　　　180　　　185　　　190　　　195　　　200　　　205　　　210
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210 215 220 225 230 235 240 245
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Cd36 →

210 215 220 225 230 235 240 245
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CD36 →

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606

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245 250 255 260 265 270 275 280
Met Ile Asn Gly Thr Asp Ala Ala Ser Phe Pro Pro Phe Val Glu Lys Ser Gln Val Leu Gln Phe Phe Ser Ser Asp Ile Cys Arg Ser Ile Tyr Ala Val Phe Glu
Cd36 →

245 250 255 260 265 270 275 280
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Cd36 →

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607

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Cd36 →

280 285 290 295 300 305 310 315
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CD36 →
Cd36 →

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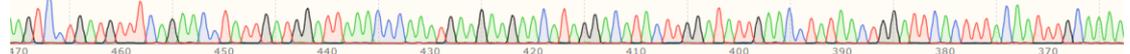
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315 320 325 330 335 340 345 350
Glu Lys Ile Ile Ser Lys Asn Cys Thr Ser Tyr Gly Val Leu Asp Ile Ser Lys Cys Lys Glu Gly Arg Pro Val Tyr Ile Ser Leu Pro His Phe Leu Tyr Ala Ser
Cd36 →

315 320 325 330 335 340 345 350
Glu Lys Ile Ile Ser Lys Asn Cys Thr Ser Tyr Gly Val Leu Asp Ile Ser Lys Cys Lys Glu Gly Arg Pro Val Tyr Ile Ser Leu Pro His Phe Leu Tyr Ala Ser
CD36 →
Cd36 →

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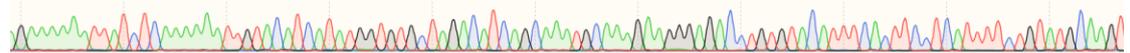
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Ser Pro Asp Val Ser Glu Pro Ile Asp Gly Leu Asn Pro Asn Glu Glu Glu His Arg Thr Tyr Leu Asp Ile Glu Pro Ile Thr Gly Phe Thr Leu Gln Phe Ala Lys
Cd36 →

350 355 360 365 370 375 380 385
Ser Pro Asp Val Ser Glu Pro Ile Asp Gly Leu Asn Pro Asn Glu Glu Glu His Arg Thr Tyr Leu Asp Ile Glu Pro Ile Thr Gly Phe Thr Leu Gln Phe Ala Lys
CD36 →
Cd36 →

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610

S5 Fig. The full-length Sanger sequencing data of the CD36 CDS in homozygous

hCD36 mice.